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Maize for production of recombinant proteins: fundamental and practical considerations

Lorena Beatriz Moeller
Iowa State University

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Maize for production of recombinant proteins: fundamental and practical considerations

by

Lorena Beatriz Moeller

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Plant Biology

Program of Study Committee:

Kan Wang, Major Professor

Diane Bassham

Harry Horner

Martha James

Gregory Phillips

Paul Scott

Iowa State University

Ames, Iowa

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ABSTRACT

The present work addresses fundamental and practical considerations of using maize for production of recombinant proteins. Maize expressing the B subunit of the heat labile enterotoxin (LT-B) of *Escherichia coli* was used as a model crop. To address the fundamental aspects of the system, the role of LT-B and its native signal peptide (BSP) in plant cells was investigated using translational fusions with the green fluorescent protein. Confocal microscopy showed that fusions carrying BSP or a maize 27 kD γ -zein signal peptide (ZSP) accumulated in the secretory system of Arabidopsis and maize. Fusions lacking a signal peptide accumulated in the cytosol or nucleus. Understanding how plants process bacterial signal sequences can aid in developing effective strategies for producing recombinant proteins in plants.

To address the practical aspects of the system, fractionation of transgenic LT-B maize by wet milling was performed using traditional (SO₂+lactic acid) or water steeping. Functional LT-B was found at highest concentration in the fine fiber, lowest in germ and starch, and with highest total LT-B recovery from water steeping. Wet milling of LT-B maize with water steeping is suitable for enrichment of a high-value protein in fine fiber; co-utilization of germ and starch for traditional uses is feasible.

A continuous *in vitro* endosperm callus culture (ENC) of Hi II maize was developed for functional analysis of seed-specific expressing genes. Morphological and biochemical characterization of ENC showed presence of aleurone and starchy endosperm cell types that resemble those of developing endosperms. ENC cells synthesize starch, zein storage proteins, and possess active starch metabolism enzymes. While the current ENC needs improvement for effective transient analysis of promoter/gene expression, it is useful for endosperm-targeted transgene analysis in stably-transformed lines of genotype Hi II.

We expressed an endo-1,4- β -glucanase from *Ruminococcus albus* in maize for potential use in bioethanol production. Molecular characterization showed successful transgene integration in recovered lines. In-depth biochemical analysis indicated that current standard assay systems need improvement for thoroughly characterizing recombinant endoglucanase.

This work illustrates that maize, an important food, feed and feedstock crop, can be used for efficient production of recombinant proteins in seeds.

CHAPTER 1: INTRODUCTION

General Introduction

Plants play a central role in life on Earth; they harvest energy from the sun and convert it into chemical energy that feeds the world. Humans have used plants for thousands of years for food, feed, clothing, medicinal and other applications. The origin of agriculture led to domestication of plant species that have been used since then to keep up with population growth and food demand. Domestication came about as a result of cultivation and selection processes that resulted in modification of the plant's genome for particular traits (Khush, 2001). The late 20th century was witness to the Green Revolution, a remarkable event in which rice and wheat varieties were bred for modified plant architecture resulting in unprecedented yield increases. Advances in genetic engineering and tissue culture of plants have opened a new window for plant modification and improvement. Genetic engineering of plants is done routinely for several species, including the most consumed crops of the world such as wheat, rice and maize.

Expression of recombinant proteins in plants continues to be an area of great interest in the pharmaceutical, industrial and vaccine industries (Horn et al., 2004). The production of recombinant proteins in crop systems, in particular, represents a potential means for commercial level production of proteins of interest in the future due to the attractive features of scalability, safety and stability. The production of recombinant proteins in maize for commercialization has been shown to be a feasible option and an ideal system for molecular farming (Ramessar et al., 2008). Though many studies have investigated and documented the production of recombinant proteins in maize, very few papers have focused on the basic understanding of recombinant protein traffic within the cell. Some studies have reported that yields of recombinant protein production in maize are related to subcellular localization (Hood et al., 2003; Streatfield et al., 2003), and few papers have shown detailed information on recombinant protein deposition in the maize kernel (Chikwamba et al., 2003). However, there exists little to no information on how plants utilize their protein sorting machinery to sort and process bacterial proteins and bacterial signals. In this study we use the B subunit of the heat labile (LT-B) enterotoxin from *Escherichia coli* (*E. coli*) as a model protein to study

subcellular targeting in a heterologous system, maize. LT-B has been produced in maize and shown to be effective in protecting mice (Lamphear et al., 2002; Chikwamba et al., 2002a) and humans (Tacket et al., 2004) against traveler's diarrhea causative agent, the enterotoxigenic strain of *E. coli*. Better understanding of how the bacterial protein is processed in plant systems can aid in future design strategies using LT-B as a carrier molecule.

In the second study we assessed the suitability of traditional maize fractionation processes for enrichment of LT-B protein. We evaluated wet milling fractionation using traditional (with lactic acid and SO₂) and water steeping conditions, and the effect on LT-B recovery in the different fractions. Wet milling is a standard operation for recovery of useful fractions of maize. The most valuable fractions of the process are the germ and starch, and the wet milling fractionation procedure is geared towards enhancing yield of these two fractions (Johnson, 2000). Fractionation of transgenic maize expressing recombinant proteins has been done mostly for germ (Kusnadi et al., 1998; Tacket et al., 2004) or endosperm (Zhong et al., 2006) targeted proteins using dry milling or hand fractionation. Preliminary studies of fractionation of maize expressing recombinant β -glucuronidase (Yildirim et al., 2002) and recombinant LT-B (Vignaux and Johnson, 2006) using wet milling suggested detrimental effects on recombinant proteins. This work focused on testing wet milling and for the potential recovery of an LT-B enriched fraction that can be used for oral delivery, downstream processing or purification of LT-B, and use of spent fractions for their traditional applications.

Expression of recombinant proteins in the endosperm of maize has several inherent limitations such as the lengthy period from transformation to availability of tissue for study, and the limited time the tissue is amenable to manipulations required in testing of the transgene of interest. With the motivation of overcoming some of these difficulties, we evaluated the possibility of establishing and using an endosperm-derived tissue culture using hybrid Hi II maize. Characterization of the culture included analysis of cell culture morphology as well as their ability to synthesize zein storage protein and active starch metabolism enzymes. Other aspects of the characterization included assessment of the utility

of the culture for promoter and transient gene expression analysis and the use of the culture to study transgene expression, when started from endosperms of transgenic maize.

Finally, we evaluated several methods to characterize transgenic maize expressing recombinant endo-1,4- β -glucanase. Standard molecular protocols such as Southern blot and the polymerase chain reaction confirmed the presence of the transgene. Two methods of analysis were tested for characterization of recombinant endo-1,4- β -glucanase activity. The first method was based on hydrolysis of carboxymethylcellulose and release of reducing sugars, and the second is based on hydrolysis of 4-methylumbelliferylcellobioside and release of fluorescent 4-methylumbelliferone.

Our studies cover fundamental and practical aspects of utilization of maize as a platform for production of recombinant proteins. Relevant information related to recombinant protein processing, transgenic maize fractionation, and recombinant enzyme characterization will provide valuable insights for future design strategies.

Dissertation organization

This dissertation is a compilation of research projects geared towards studying different aspects of recombinant protein production in plants, and specifically, in maize. The chapters included in this thesis describe my work as a student under the guidance of Dr. Kan Wang.

Chapter 1 comprises a general introduction, a description of the dissertation organization and a literature review. The literature review includes topics relevant to all the projects presented in the dissertation. I cover topics related to plant biotechnology such as its applications, limitations and future directions. I also address some advantages of using maize as a production system for recombinant proteins, and present several examples of the use of maize as a biofactory.

Chapter 2 is a reprint of a scientific review article that has been published in the peer reviewed journal, BioScience, as a cover featured paper in May 2008. The title of the article is “Engineering with precision: tools for the new generation of transgenic crops”. I co-authored the review with Dr. Kan Wang.

Chapter 3 describes the research conducted towards studying the subcellular localization and trafficking of LT-B in *Arabidopsis thaliana* and maize using the green fluorescent

protein (GFP) as a visual marker. The manuscript has been submitted for publication in Journal of Experimental Botany. I designed the experimental approach, gene constructs, Arabidopsis transformation, molecular analysis and imaging of transgenic lines, ELISA determinations and starch and fiber isolations. Qinglei Gan assisted in performing western blots analysis and thermolysin treatments of starch.

Chapter 4 describes the work done to determine the utility of wet milling for fraction enrichment of maize-derived LT-B. This chapter has been submitted for publication in Biotechnology Progress. This work investigates the effect of traditional steeping conditions in LT-B recovery from transgenic maize. The work presented in this chapter was a collaboration between the Wang Lab and the Center for Crops Utilization Research at Iowa State University. Steve Fox was in charge of performing the wet milling fractionation of maize. Raye Taylor-Vokes analyzed the fractions obtained by Steve Fox for initial screening by ELISA. Qinglei Gan was in charge of running protein gels and western blots. I completed fraction analysis by ELISA, analyzed the data and wrote the manuscript for publication. Drs. Larry Johnson and Kan Wang provided significant materials for the discussion and publication.

Chapter 5 presents the work towards establishing a continuous endosperm callus culture of maize, its characterization and potential applications. This chapter is a manuscript prepared to be submitted to Plant Cell Reports. For this work, I was in charge of initiation of cultures, initial protein characterization, semi-quantitative PCR, tissue embedding and staining, transient gene expression analysis and fluorescent imaging. Qinglei Gan was instrumental in establishing zymogram analysis and later stages of protein western blots.

Chapter 6 presents the initial attempts to characterize transgenic maize lines expressing an endo-1,4- β -glucanase from *Ruminococcus albus* using published methods of endoglucanase assay. I did all molecular characterizations, initial glucanase activity screening of transgenic materials, training of all other people involved in the project and compilation of the results. Mahwish Hafeez established the carboxymethylcellulose-microplate-based assay and analyzed seed-specific events. Qinglei Gan established the methyl-umbelliferyl-cellobioside assay, and also was instrumental in assessment of utility of the CMC- assay. Hongxin Jiang studied the degree of hydrolysis of CMC by transgenic

extracts and performed the sepharose gel chromatography. Drs. Jay-lin Jane and Kan Wang provided significant inputs for experimental approaches and interpretation of results.

Finally, Chapter 7 presents the general conclusions for the dissertation. Included as appendices are two publications in which I was co-author. Appendix 1 is a review article titled “Genetic engineering approaches to improve bioethanol production from maize” published by invitation in the series *Current Opinion in Biotechnology* in 2007. As a second author in this publication I contributed about 30% of the efforts and materials in the manuscript. I was particularly active in discussing the modification of starch and yield grain enhancement through genetic engineering. Appendix 2 includes a scientific paper titled “Localization of a bacterial protein in starch granules of transgenic maize kernels” published in the *Proceedings of the National Academy of Sciences, USA*, in 2003. For this manuscript I was third author and generated the data used for Table 1, Fig. 4 and Fig. 5 that include percentage of total protein and LT-B in transgenic maize kernel fractions and experiments to determine the thermal stability of LT-B in transgenic maize ground meals and purified starch.

Literature review

Plant biotechnology

Biotechnology refers to the use of biological systems or processes for human benefit. Plant biotechnology, thereof, refers to the use of plants, plant parts or plant products for our benefit (Boulter, 1995). Utilization of plants has been inherent to human practices since the beginning of human agriculture. Applications of plant biotechnology in the early days included but were not limited to utilization of plants and plant extracts for medicinal purposes, use of plant fibers for clothing and construction, use of plants and microorganisms for bread and beer making, and breeding of crop plants for enhanced agronomic traits. Even though human efforts in plant breeding have been continuous for the past 10,000 years (Schlegel, 2007), selection for desirable traits was empirical for a long time. Following the discovery of Mendel’s laws of inheritance in the early 19th century, the 1960’s was a fruitful decade in which breeding of maize, rice and wheat varieties with enhanced agronomic performance resulted in an unprecedented increase in food production known as the Green Revolution (Khush, 2001; Hedden, 2003). Many breeding techniques such as hybrid

breeding, mutation breeding, chromosome substitutions and translocations and somaclonal variation using *in vitro* culture, among others, are examples of plant biotechnology (Jauhar, 2006).

Plant genetic engineering allows introduction of genes into plant genomes using recombinant DNA technology. Genetic engineering allows the introduction and expression of genes from distant species into the plant or crop of choice in a controlled fashion. Plants carrying genes from a different species, or transgenes, are referred to as transgenic plants. In simple terms biotechnology has been described as a scientific method that enables precision in developing particular genotypes (McLaren, 2005).

Transgenic plants are used nowadays in many distinct applications, from basic science to molecular farming. Transgenic plants in basic science continue to be a tool for studying different aspects of gene function and regulation. From an agronomic standpoint, transgenic plants are designed for specific output traits including pest (Dempsey et al., 1998) and herbicide resistance (Greef et al., 1989; Cao et al., 2004), resistance to environmental stress conditions (Kasuga et al., 1999; Sanan-Mishra et al., 2005), enhanced yield (Smidansky et al., 2002), improved nutritional value (Díaz de la Garza et al., 2004; Diaz de la Garza et al., 2007; Delaunois et al., 2009), improved shelf-life (FDA/CFSAN, 1994), etc. Value added traits include the generation of transgenic plants for the production of pharmaceuticals (Goldstein and Thomas, 2004; Vitale and Pedrazzini, 2005) or industrial enzymes (Taylor Li et al., 2008) and products (Suriyamongkol et al., 2007), potential for phytoremediation (Gleba et al., 1999; Kramer, 2005) and improved feedstocks for biofuel production (Sticklen, 2006; Dhugga, 2007), among others.

Advances in plant genetic engineering have come hand in hand with improvement in plant tissue culture and plant transformation protocols. Studies in plant growth and development have allowed researchers to use different hormones in tissue culture protocols to successfully grow many species in cell culture. At the same time, substantial work done in transformation technologies has allowed transformation of species originally recalcitrant to the process. Today we are able to transform the most important crop species to human population, namely cereals (Rasco-Gaunt et al., 2000), including maize (Gordon-Kamm et

al., 1990; Frame et al., 2000), rice (Li et al., 1993; Enríquez-Obregón et al., 1999), wheat, barley and oats (Dunwell, 2009).

Maize as a bioreactor for production of high value proteins

Why maize?

Maize has played a central role in the lives of people of the Americas for thousands of years. Domesticated maize grown in Mexico's valley of Tehuacan as early as 5,000 B.C. evolved from its wild grass relative, Teosinte, *Zea mays* spp. *parviglumis* through human selection and genetic manipulation (Doebley, 2004). By selecting plants with patterns of growth and development that enhanced food production, early farmers began the domestication process through artificial selection on particular alleles or gene combinations that regulated the desired characteristics (Vollbrecht and Sigmon, 2005). Domestication along with improvement of crops and agronomic practices through a combination of techniques has resulted in an unprecedented increase in crop yield we know as the green revolution (Khush, 2001). In Mayan cultures maize was staple food and it also had a religious meaning. Early colonizers in the United States learned from Native Americans how to process the maize grain into food and feed. With development of processing procedures, maize uses began to diversify widely in the food, feed and industrial applications (Johnson, 2000).

The maize kernel is a natural site of protein and nutrient storage and is an attractive target for biotechnological applications. Endosperm and germ compose 83% and 11% of a maize kernel, respectively (Johnson, 2000). The major chemical components in the endosperm fraction are starch (88%) and protein (8%) while in the germ it is fat (33%), protein (18%) and starch (8%). Maize transformation has come to change the shape of the world's economy in the last decade. About 24% of the maize grown today in the world is transgenic (Brookes and Barfoot, 2006). Maize transformation and tissue culture are highly established practices (Frame et al., 2000; Frame et al., 2002) and successful stable transgenic maize plants have been developed for expression of a variety of recombinant proteins. Maize has proven to be an effective expression system for functional proteins of prokaryotic, viral and eukaryotic origins. The commercialization of β -glucosidase (Witcher et al., 1998), aprotinin

(Zhong et al., 1999) and avidin (Hood et al., 1997) demonstrate the viability of this expression system for commercial purposes. There are several benefits of producing recombinant proteins in maize. Maize is staple food and feed crop around the world, with established production systems and high yield practices. Maize seeds are natural protein storage reservoirs that have been used for recombinant protein accumulation in the past. Breeding programs used for agronomic and food production can also be used in transgenic maize to enhance the production of recombinant proteins. Storage and transport of transgenic dry grain can be accomplished as for regular maize and many recombinant proteins have shown to be stable in the dry kernels for extended periods of time. Efficient production of recombinant proteins in maize can help reduce costs of production, storage and processing. From a technical standpoint, maize also offers the advantage of possessing the necessary machinery for post-translational modifications of proteins, which influences to great extent the activity and nature of the recombinant protein of interest (Faye et al., 2005). Numerous reviews have summarized the potential use of maize as a bioreactor for production of recombinant proteins (Kusnadi et al., 1998; Hood et al., 1999; Streatfield et al., 2003; Ramessar et al., 2008) and biofuel potential (Dhugga, 2007; Torney et al., 2007).

Choice of maize tissue

Recombinant proteins produced in maize kernels can be directed to specific compartments within the seed. Expression can be directed to the endosperm using endosperm specific promoters such as the zein promoters (Chikwamba et al., 2002b; Shepherd et al., 2008a, b) or to embryo (germ) by using constitutive promoters such as CaMV 35S (Chikwamba et al., 2002b), maize ubiquitin (Streatfield et al., 2002) or maize globulin1 (Shepherd et al., 2008a, b) promoters. Successful transgenic protein expression is driven by selection of specific promoters and targeting the proteins to specific compartments within the cell. Advantages of recombinant protein expression in germ are that it is rich in soluble proteins that are stable during storage; it can be separated from other seed tissues to concentrate proteins and reduce dose size. Expression in the endosperm, on the other hand, may allow higher yield and ease of protein extraction, especially if the protein of interest can be separated together with the starch fraction. The maize endosperm has been studied for several decades now, and more recently, has been the focus of biotechnological approaches

to diversify its use by humans. Efforts have been made to alter the endosperm's protein composition (Crow and Kermicle, 2002) and starch quality and digestive properties (Ao et al., 2007).

Endosperm tissue culture

Studies of developmental and cellular mechanisms of maize seed are traditionally carried on using available mutants or using forward or reverse genetics. Both of these sources of material involve a lengthy and laborious process. Analysis of endosperm is limited then, to the duration of endosperm filling, and manipulations are limited due to the nature of the endosperm tissue which makes it hard to work with. In order to overcome some of the difficulties associated with limited endosperm tissue availability and amenability to manipulation, endosperm tissue culture has been the focus of extensive research for decades. From the initial report of *in vitro* growth of maize endosperms as early as 1949 (La Rue, 1949), maize endosperm tissue cultures have existed for some time and continue to yield novel findings. Utilizing LaRue's original A636 endosperm culture, researchers have established culture requirements (Straus and La Rue, 1954; Shannon and Liu, 1977), reported spontaneous changes *in vitro* (Straus, 1958) and studied the ultrastructure of the cells (Felker, 1987). Other groups studied the effect of inbred and hybrid genotypes on culture establishment (Shannon and Batey, 1973), anthocyanin production (Racchi and Manzocchi, 1988), sugar uptake (Felker and Goodwin, 1988), zein expression (Shimamoto et al., 1983), and glycoprotein synthesis (Riedell and Miernyk, 1988) of the cultured endosperm cells. Endosperm cultures have been used for transient transformation analysis of zein gene regulation and chimeric gene expression (Quayle et al., 1991; Ueda and Messing, 1991) and most recently were used for study of aleurone cell specification in maize (Gruis et al., 2006). These applications of maize endosperm culture as a tool to study gene expression and cell signaling make it valuable for future research.

Maize fractionation

Maize fractionation is traditionally done using dry or wet milling procedures. Dry milling with degerming allows recovery of hulls, germ-rich and endosperm-rich fractions by physical and mechanical means. On the other hand, wet milling results in recovery of six

fractions including germ, gluten meal, pericarp, coarse fiber, fine fiber and starch, and steep water (Johnson, 2000). While dry milling procedures are good for separating the germ and recovering endosperm with lower oil and fiber content, wet milling offers the advantage of recovering other fractions such as highly purified starch that can be used for diverse applications. Traditional wet milling involves steeping with some combination of SO₂ and lactic acid for higher purity and yield of starch fraction (Haros et al., 2004). Fractionation studies for recovery of recombinant proteins have been carried out for germ-targeted (Hood et al., 1997; Kusnadi et al., 1998; Yildirim et al., 2002; Shepherd et al., 2008a, b) and endosperm-targeted proteins (Zhong et al., 2006; Shepherd et al., 2008a, b; Zhang et al., 2009) mainly using dry milling or other non-wet milling procedures. The utility of wet milling for recombinant protein recovery is of interest to the community due to the increased number of fractions it produces, and the different potential applications for each.

Heat labile enterotoxin from Escherichia coli (E. coli) as a model for edible vaccine production in plants

The heat labile enterotoxin (LT) of *E. coli* is the causative agent of the common disease called traveler's diarrhea, a disease similar to but less severe than the sometimes deadly cholera disease caused by *Vibrium cholerae* bacterium. Even though the nucleotide sequences of the cholera toxin (CT) and the LT have close to 80% homology, the symptoms associated with each of them can vary and might be related to different secretion and processing pathways in the bacteria (Spangler, 1992). Both CT and LT are heterohexameric proteins of about 84 kDa in size, and are toxins produced by bacteria residing in the lumen of its host's intestines. The toxins are composed of an A subunit and a B subunit, joined together by a polypeptide linker. The B subunit is a homopentameric ring that binds specifically to ganglioside G_{M1} in the membranes of the intestinal cells. The subunit A is then internalized into the cytosol where it is reduced and the resulting A1 fragment goes on to bind NAD and catalyze the ADP ribosylation of a GTP-binding protein associated with adenylate cyclase, Gs α . The resulting increase in cyclic AMP and synthesis of prostaglandins results in accumulation of salt and water in the intestinal lumen leading to the cramps and diarrhea that characterize the disease. The separate subunits alone are not

cytotoxic, due to the fact that the immunogenic and toxic effects are in separate subunits. A comprehensive review describes the structure and function of both toxins (Spangler, 1992).

LT-B production in plants for edible vaccines

Because of its properties as mucosal adjuvant and its lack of toxicity, the B subunit of LT (LT-B) has been produced in several plant systems for possible utilization as vaccines. LT-B has been expressed in potato (Mason et al., 1998), tobacco (Kang et al., 2003), maize (Chikwamba et al., 2002a; Tacket et al., 2004) and soybean (Moravec et al., 2007). The expression of the the B subunit of the heat labile enterotoxin (LT-B) of *E. coli* in maize has been accomplished by using promoters for expression and accumulation in different maize tissues. Endosperm-specific promoter leads to the LT-B production specifically in the seed endosperm tissue (Chikwamba et al., 2002b), while constitutive promoter results in embryo (or germ) enriched LT-B expression (Streatfield et al., 2002). Maize-derived LT-B from embryo and endosperm expression has been shown to be an effective orally delivered antigen in mice and humans for protection against traveler's diarrhea caused by enterotoxigenic strains of *E. coli* (Lamphear et al., 2002; Chikwamba et al., 2002a; Tacket et al., 2004).

Use of LT-B as a carrier molecule

Due to the highly antigenic nature of LT-B (Beyer et al., 2007) and its characteristic recognition by the G_{M1} receptor in the host epithelial cells, several groups have tried to generate fusions of LT-B with other proteins for co-delivery to mucosal surfaces via G_{M1} internalization. The use of LT-B as a carrier has been extensively studied in bacterial systems (Cardenas and Clements, 1993) but reports of plant-derived LT-B fusions are few. Fusions of small molecules (5 – 6 kD) to the carboxy terminus of LT-B (Rigano et al., 2004; Rosales-Mendoza et al., 2009) have been reported. In both of these cases, the peptides used in the fusion represent about 1/2 of the size of the LT-B protein itself, which limits the number of proteins that can be used for co-delivery while having the proteins retain their native conformations and functionalities.

LT-B translocation and subcellular localization

The subcellular localization of a protein accounts greatly for its proper function within the cell. Complex systems that allow compartmentalization of distinct proteins in different organelles have evolved in nature to provide the specialization of the eukaryotic organelles. Elucidation of these mechanisms combined with the controlled regulation of gene expression for recombinant proteins can be a powerful tool. The cellular machinery has evolved to become a streamlined delivery system of resident proteins to their intended locations (Schatz and Dobberstein, 1996; Agarraberes and Dice, 2001).

Protein trafficking and translocation in bacteria and plant cells

Protein translocation pathways in bacteria (Papanikou et al., 2007; Driessen and Nouwen, 2008) and plants (Raikhel and Chrispeels, 2000; Hanton et al., 2007; Hormann et al., 2007; Brown and Baker, 2008) have been extensively studied and reviewed. Fundamental differences and some common themes exist since trafficking of proteins often includes translocation across membranes (Schatz and Dobberstein, 1996; Agarraberes and Dice, 2001). In Gram-positive bacteria secreted proteins only need to cross the plasma membrane, while in Gram-negative bacteria, secreted proteins are translocated through the plasma membrane and the outer membrane. Most secreted proteins carry an N-terminal signal sequence for targeting to the inner membrane protein translocation machinery to be internalized into the membrane, translocated to the periplasm or secreted (Saier, 2006; Saier et al., 2008). The sorting of proteins in plant cells involves a more complex ‘address system’ to be able to target different organelles. Plant cell protein translocation, hence, involves different types of membranes. An N-terminal signal peptide often directs proteins for secretion, transported into the endoplasmic reticulum (ER) lumen, and sorted thereafter to the Golgi, cell wall, vacuole or plasma membrane, or retained in the ER and ER-derived protein bodies. Proteins lacking an ER signal sequence translated in the cytosol, are then maybe translocated into other organelles such as the plastids (Inaba and Schnell, 2008; Paul, 2008) or mitochondria (Attardi and Schatz, 1988) if carrying appropriate targeting signals. A novel trafficking pathway has been described between the secretory pathway and the plastids (Villarejo et al., 2005; Nanjo et al., 2006). Two native plant proteins, α -carbonic anhydrase

from *Arabidopsis* (Villarejo et al., 2005) and rice plastidial N-glycosylated nucleotide pyrophosphatase/phosphodiesterase (Nanjo et al., 2006) were shown to localize to the plastids after being processed in the endoplasmic reticulum.

Recombinant protein trafficking and subcellular localization in plant cells

Though much has been learned about the translocation machineries of bacteria and plants, less is known on how plants utilize their molecular translocation machineries to sort bacterial proteins when expressed in transgenic plants. LT-B has a native signal peptide that directs the protein for secretion in bacteria. The secretion mechanism used by enterotoxigenic strains of *E. coli* (ETEC) to deliver LT to the intestinal lumen resembles type II secretion in *Vibrio cholerae*. The A and B subunits of LT carry N-terminal signal peptides that are cleaved during Sec-dependent translocation into the periplasm (Tauschek et al., 2002). After folding and assembly, the holotoxin is transported across the outer membrane via a type II secretion pathway present in ETEC strains, but absent in certain laboratory strains such as *E. coli* K-12 (Tauschek et al., 2002). Once secreted, LT binds lipopolysaccharide via a G_{M1} independent binding region on the B- subunit and is assembled in vesicles, free to bind G_{M1} in the host's cells (Horstman and Kuehn, 2002).

LT-B has been expressed in several plant species such as potato (Mason et al., 1998; Tacket et al., 1998), tobacco (Kang et al., 2003), maize (Chikwamba et al., 2002b; Streatfield et al., 2003) and soybean (Moravec et al., 2007). Targeting of LT-B to different organelles in plant cells has been shown to affect its accumulation dramatically (Streatfield et al., 2003). Though several of these studies describe the targeting of LT-B to different cellular compartments by the inclusion of targeting signals within their constructs (Streatfield et al., 2003), only two studies show detailed subcellular localization of LT-B in the transgenic material (Chikwamba et al., 2003; Moravec et al., 2007). Streatfield and colleagues (Streatfield et al., 2003), for example, used the signal sequences from barley alpha amylase and aleurin to target LT-B to the cell surface and vacuole, respectively, and the maize granule-bound glycogen synthase to target LT-B to the plastids. The greatest amount of LT-B was detected when it was targeted to the vacuole and the least amount was detected when it was targeted to the plastid and cytoplasm. Subcellular localization of LT-B in transgenic plants is not straight-forward. In the Chikwamba work the functional LT-B was found in the

starch granules of transgenic maize kernels expressing the LT-B with its native bacterial signal peptide. In the Moravec work the replacement of bacterial signal peptide with an *Arabidopsis* basic chitinase signal peptide at the N-terminus of LT-B resulted in the localization of LT-B in protein bodies of transgenic soybean seeds.

The green fluorescent protein (GFP) as a visual marker in plant cells

The green fluorescent protein (GFP) from *Aequorea victoria* jellyfish, is a popular reporter gene used widely in biological systems (Mankin and Thompson, 2001). Several variants of GFP have been generated using mutagenesis procedures in order to enhance and improve their applications to plant systems (Mankin and Thompson, 2001). Some of the advantages that make GFP a preferred reporter protein over commonly used luciferase (LUC), is that GFP does not require addition of a substrate (which can be difficult to deliver due to impermeability of plant cells) and that it allows visualization at subcellular level (Haseloff et al., 1997). The main advantage of GFP is considered to be the fact that it allows visualization of cells or organisms *in vivo*. Original versions of GFP used in plants had limited expression due to abnormal mRNA splicing and low solubility. Codon optimization for expression in *Arabidopsis thaliana* (Haseloff et al., 1997) and increased solubility (Davis and Vierstra, 1998) resulted in increased fluorescence *in planta*. Other mutations such as replacement of the serine at position 65 with a threonine in the chromophore (S65T variant) combined with codon optimization have also resulted in more than 100-fold brighter fluorescent signals upon excitation with 490 nm (blue) light (Chiu et al., 1996). GFP has been used to visualize tissue specific activity of promoters (Shepherd et al., 2008a), targeting properties of signal sequences and proteins (Cutler et al., 2000), and recently, as a reporter for tissue separation during grain processing (Shepherd et al., 2008b), among many other applications which have been extensively reviewed (Chiu et al., 1996; Haseloff and Siemering, 1998; Hanson and Kohler, 2001).

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CHAPTER 2: ENGINEERING WITH PRECISION: TOOLS FOR THE NEW GENERATION OF TRANSGENIC CROPS

Lorena Moeller and Kan Wang

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Abstract

In the past 25 years, a major revolution in agricultural practice and crop production has occurred. Genetically engineered crops with improved agronomic traits have made the transition from laboratory benches and greenhouses to fields all over the world, where they are being grown commercially. Genetic engineering technologies have evolved as a science and continue to provide the tools for making the crops of tomorrow. Armed with genomic information and nanotechnology, plant molecular biologists are redesigning molecular tool kits to engineer plants with more precision. This article describes the current major transformation methods, discusses their strengths and limitations, and focuses on a number of research areas that are likely to be used for producing the new generation of transgenic crops.

Introduction

Human efforts in plant breeding have been continuous for the past 10,000 years (Schlegel 2007). Our ancestors have been altering the genetic makeup of crops by selecting for desirable features such as faster growth, larger seeds, or sweeter fruits. As a result of this lengthy selection process, domesticated plant species can be drastically different from their wild relatives. Although such efforts have contributed significantly to the food supply and stability of human society, most modern crops were created with little knowledge of the genetics and fundamentals of modern plant breeding. The 20th century has seen major advances in crop development, following the discovery of Mendel's laws of inheritance. In the 1960s, the breeding of maize, rice, and wheat varieties with better yield potential, disease and stress resistance, and adaptability to different environments resulted in an unprecedented increase in food production that came to be known as the Green Revolution (Hedden 2003).

Taking valuable traits and carefully incorporating them into future generations has enabled breeders to produce better crops that possess the desired combination of traits (Figure 1A). Many breeding techniques (such as hybrid breeding, mutation breeding, chromosome substitutions and translocations, somaclonal variation using *in vitro* culture) have been developed to enhance the discovery, selection, and integration of important agronomic traits (Jauhar 2006, Schlegel 2007). Although these methods have been useful in generating improved species, they are limited in correlating traits with specific gene functions—that is, the introduction of a desired trait to a recipient line often leads to the introduction of numerous undesired traits. These breeding methods often require lengthy selection and elimination processes to produce the desired traits and get rid of undesired ones. In addition, it is not possible to introduce genes from a distant species.

Genetic engineering technology, the most recent method for advanced breeding, introduces genes with known functions into plant genomes for crop improvement through recombinant DNA (deoxyribonucleic acid) technology. It not only provides the means for the introduction and expression of genes from distant species (Figure 1B) but also allows controlled gene expression (such as developmental or tissue-specific expression). We are not yet able to predict and control the molecular interactions and outcomes of introduced transgenes and endogenous genes. However, we can develop a crop expressing an enhanced trait associated with a specific gene more effectively than we could before.

The gene revolution: A quick look back

Genetically engineered (GE) or transgenic crops are generated by altering the crop's genetic makeup (i.e., introducing, deleting, or silencing a gene or group of genes of interest) using recombinant DNA technology. Whereas transgenic plants are generated by insertion of heterologous DNA sequences from a different species, cisgenic plants are generated by re-introducing or silencing a gene of the same species, usually under regulation of the native elements (Schouten et al. 2006). Today, most GE plants—if not all—can be referred to as transgenic plants because various heterologous DNA sequences are present, carried over through the engineering process. In this article, the term “transgenic” will be used to refer to both transgenic and cisgenic crops.

The generation of transgenic plants was first reported in 1983 Miami Winter Symposia (Advances in Gene Technology: Molecular Genetics of Plants and Animals) by three independent groups from the University of Ghent in Belgium, Washington University, and Monsanto Company. These groups subsequently published their results on transgenic tobacco resistant to kanamycin and methotrexate (Herrera-Estrella et al. 1983), on kanamycin-resistant *Nicotiana plumbaginifolia* (a close relative to ordinary tobacco; Bevan et al. 1983), and on kanamycin-resistant petunia plants (Fraley et al. 1983), respectively.

In 1987 came a report of the successful genetic engineering of tobacco plants expressing a chimeric *Bacillus thuringiensis* toxin encoded by the *bt2* gene, and showing resistance to lepidopteran insect attack (Vaeck et al. 1987). This was the first report of an agronomically relevant trait incorporated into the plants and evaluated. Even though tobacco is not a major food crop species, the transgenic tobacco plants resistant to insect damage marked the beginning of a new era in genetic engineering.

In 1994, after three years of evaluations by the Food and Drug Administration (FDA), the Californian company Calgene was allowed to commercialize the first transgenic crop for food consumption, the Flavr Savr Tomato. The Flavor Savr tomato was designed to delay ripening on the vine, allowing more flavor to build in the fruit before harvesting and extending the fruit's shelf life (www.cfsan.fda.gov/~lrd/biotech.html). Because of various technical difficulties associated with transgenic tomato production, as well as consumer concerns about the presence of an antibiotic selectable marker gene in the plants, Flavr Savr tomatoes were removed from the shelves after only a couple of years on the market. However, by 1995, applications had been made to the FDA for review and approval of various new GE crops, including virus-resistant squash; potatoes resistant to the Colorado potato beetle; and herbicide-tolerant soybean, corn, canola, and cotton (www.cfsan.fda.gov/~lrd/biocon.html).

The boom in the era of plant genetic engineering can be appreciated by comparing the numbers of species and permits for regulated-article field release in the last 20 years. In 1987, tomato and tobacco were the only organisms with field release permits, with a total of five permits. In 2007, the US Department of Agriculture (USDA) and its Animal and Plant Health Inspection Service (APHIS) unit authorized a total of 671 field release permits

(www.nbiap.vt.edu/cfdocs/fieldtests1.cfm) for 54 organisms and several phenotype categories (Figure 2). According to reports published by the International Service for the Acquisition of Agri-biotech Applications, 102 million hectares of GE crops were planted worldwide in 2006, a 60-fold increase since 1996 (Brookes and Barfoot 2006). This figure makes biotechnology the fastest adopted crop technology in recent history. In 2005 alone, the economic benefit of GE crop adoption to farm income was about \$5 billion (Brookes and Barfoot 2006). The biotech generation of crops has resulted in a 15.3% net reduction in environmental impact on GE crop farming land since 1996, owing in great measure to significant reductions in the application of herbicides and insecticides worldwide. It is estimated that more than 10 million farmers have adopted GE crop technology and have experienced the benefits and profits that biotechnology can bring (Brookes and Barfoot 2006).

For basic research purposes, transgenic plants are generated to understand gene function and regulation mechanisms. Over-expression or down regulation of key enzymes or regulatory factors often perturb known or unknown pathways that can lead to significantly altered phenotypes. For crop improvement purposes, transferred genes usually confer an output trait that renders the transgenic plant superior to its non-transgenic counterpart. Desirable output traits include pest and herbicide resistance; resistance to environmental stress conditions; enhanced yield; improved nutritional value; improved shelf life; altered oil, sugar or starch contents; production of plant-based pharmaceuticals or industrial products; and potential for bioremediation.

The term “genetically modified organism” is frequently used to describe transgenic crops. In fact, all crops generated and cultivated through conventional breeding have been genetically modified as well. Therefore, “genetically engineered” or “biotech” should be used to more precisely describe transgenic crops created through biotechnology.

Smart bug and top gun: Delivering genes into plants

The revolution in genetic engineering of plant species can be attributed to the remarkable research progress on studying a plant pathogen, *Agrobacterium tumefaciens*. In nature, this soil bacterium infects a wide range of host plants. It transfers a gene-encoding segment DNA

(T-DNA) from its own plasmid (the circular extrachromosomal molecule in *Agrobacterium*) into the plant genome to promote production of opines, nutrients that only *Agrobacterium* is able to use for survival and reproduction. Along with the opine genes, the bacterium also transfers phytohormone genes to promote cell proliferation, resulting in the formation of tumors commonly known as crown galls.

The molecular dissection of the *Agrobacterium* tumor-inducing plasmid (Ti plasmid) allowed researchers to design a “disarmed” vector system, in which the virulence functions of *Agrobacterium* Ti plasmid were removed and replaced with a gene of interest, while retaining its DNA transfer abilities. By simply infecting plant cells or tissues with the engineered strain, the researchers were able to generate healthy transgenic plants carrying the gene of interest (Figure 3A; Gelvin 2003).

Although the *Agrobacterium*-mediated method generated numerous transgenic plants, it appeared to be ineffective for many crop species, especially during the early years of genetic engineering. To overcome this problem, many physical delivery methods have been tested. Use of plant protoplasts for methods such as electroporation, injection, or delivery of DNA using polyethylene-glycol proved successful, but efficiencies were very low, costs were high, and applications were limited to certain plant species. Other methods such as tissue electroporation and silicon carbide whisker-mediated transformation have yielded transgenic plants but have not found wide applications (Potrykus and Spangenberg 1995). The bio listic gun, a device that literally shoots tungsten or gold particles coated with the DNA of interest, came to be the delivery method of choice for species recalcitrant to *Agrobacterium* mediated transformation (Figure 3B).

The success of the transformation does not depend only on the gene delivery method. The combination of selection markers and gene regulatory elements also determines the success of both the generation of the transgenic events and the expression of the transgene of interest. The choice of appropriate target cell or tissue and the judicious treatment of the target tissue before and after transformation are important for the survival and selection of the transgenic events. The effect of the number of transgene copies on the transgene’s expression level is still an amply debated issue. Transgenic plants carrying more copies of a transgene in their genomes do not always give a high level of transgene expression. In fact, integration of high

numbers of transgene copies often leads to transgene silencing, a phenomenon in which the expression of the transgene or endogenous gene with sequence homology is partially or completely reduced in transgenic plants (Shou et al. 2004, Travella et al. 2005). Transgene transcript dosage (amount of RNA [ribonucleic acid] transcripts) is responsible for some of the silencing and variability observed in transgene expression among transgenic lines carrying the same construct (Schubert et al. 2004). Compared with the biolistic gun method of DNA delivery, *Agrobacterium*-mediated transformation results in more transgenic events with a lower or single-copy number of transgene integrations, and thus is currently the preferred system for most plant scientists.

Successes, limitations, and future outlook of current genetic engineering technology

The past 25 years have seen tremendous technological advances in plant biotechnology and rapid adoption of biotech crops in agricultural practice. At the same time, limitations associated with early transgenic technology have raised concerns about the long-term impacts that GE crops may have on health and the environment. From a scientific standpoint, the successful application of genetic engineering to more crops for more traits has been hindered in more than one way. The addition of two or more genes or desired traits into a single species of interest is known as gene stacking. Gene stacking in plant biotechnology is important because it makes the design of transgenic plants highly versatile, allowing, for example, the engineering of resistance to multiple pathogens, multi-toxin resistance, and metabolic pathway engineering (Halpin 2005). Many of the new technologies for developing transgenic plants are designed with their ability for gene stacking in mind.

Location, location, location: Overcoming transgene position

Although genetic engineering has the advantage of allowing defined segments of DNA and genes with known functions to be introduced into a genome of a distant plant species, it cannot yet control the location and numbers of the introduced gene fragments. One inherent problem with current transformation technologies is that transgenes are inserted randomly into the plant genome, and consequently each transgene is inserted into a different genomic environment that can significantly affect its expression (position effect). Furthermore,

transgene expression can vary depending on insertion-site complexity. Analysis of transgene insertion sites has shown that the transgene often integrates as multiple copies, especially when biolistics is the gene delivery method (Makarevitch et al. 2003). Transgene copies may be incomplete or arranged in direct or inverted repeats, often resulting in silencing the transgene. The position effects and insertion-site complexity that arise from the location of the transgene in the genome have been an issue of concern for plant biotechnologists since the early days of the modern biotechnology era. The current approach to solving the problem is to screen hundreds to thousands of independent transformation events for the few with simple integration structures that express the transgene at the desired level. One of the early strategies to reduce complexity problems was to bombard linear DNA molecules, yielding DNA insertions of less complexity but still random integration into the genome (Breitler et al. 2002). Shielding the transgene from the genomic environment by flanking it with matrix attachment regions (MARs) was another strategy tested. Although the technique seemed promising, the reported findings from this approach were highly variable and showed dependence on the transgene construct and the MAR used (Mankin et al. 2003).

Site-specific recombination is a strategy explored actively in plant systems to circumvent the position effect. Many variations of site-specific integration, deletion, or inversion of transgenes have been used in plants, including at least three different recombination systems: the Cre-*lox* from bacteriophage P1, the FLP/*FRT* from yeast, and INT from phiC31 *Streptomyces* phage (Ow 2005). The results of recombination depend on the initial location of the recombination site, as well as on the inherent capability of the recombination system. Flanking a transgene with directly oriented *lox* or *FRT* sites can lead to deletion of the flanked transgene, such as selectable marker genes no longer needed after transformation (Ow 2007). Flanking a transgene with inversely oriented sites can resolve multiple transgene integration patterns, as shown with the Cre-*lox* system in wheat, rice, and maize (Ow 2005). Recombination among the different integrated copies leads to the simplest recombination unit, generally a single copy that can only invert, not delete. In rice, Cre-directed site-specific integration places a precise single-copy DNA fragment into the target site in nearly half of the selected events. These precise, single-copy-insertion plants express the transgene within a range that is predictable and reproducible, indicating that once a suitable target site is found,

the plant line can be used for predictable insertion and expression of genes (Ow 2005). Recently, high-lysine corn LY308, developed by Monsanto Company using this technology, obtained USDA regulatory approval to have nonregulated status for commercial release in the poultry industry (www.aphis.usda.gov/brs/aphisdocs2/04_22901p_com.pdf).

One limitation with the current recombinase-based system is that it first requires development of well-characterized lines carrying target sites that will permit stable and predictable transgene expression. These founder lines can then be used for the introduction of a gene of interest. Because the generation of founder lines uses conventional transformation methods (biolistic or *Agrobacterium*), one needs to characterize a large transformation population to identify suitable founder lines. Nevertheless, once the founder lines are established, they can be used to precisely insert any compatible transgene construction. Inducible systems for recombinase gene Cre expression and self-excision (Ow 2007) have recently been used to accelerate the process. It is expected that further technological advances will help to create a new generation of transgenic plants with the desired insertion locations and more predictable gene-expression levels. Moreover, this technology will allow the removal of unwanted transgene sequences and improve the ability for gene stacking in transgenic plants (Ow 2005).

The use of zinc-finger nucleases (ZFNs) as molecular scissors for gene targeting was recently adapted to animal and plant systems (Durai et al. 2005, Porteus and Carroll 2005). Zinc fingers (ZFs) are highly specific, DNA-binding motifs found widely across all eukaryotic genomes. ZFNs are engineered proteins carrying highly specific ZF domains and a nonspecific nuclease domain, such as the one from endonuclease Fok I, that deliver a double-strand break at a targeted site in the genome. The generation of a double-strand break in the genome, in turn, induces homologous recombination to mediate site-specific recombination for genome repair. Unlike other forms of genome repair, homologous recombination is accurate and uses a homologous sequence (from an undamaged sister chromatid or the delivered gene of interest) as a source for repairing the DNA break. The specificity of the ZFNs comes from the ZF domain, as each of these domains recognizes specific triplets in the genome. By engineering three or more of these ZFs in tandem, one can

achieve the specificity required and potentially target every gene in the genome of most crop plants (Durai et al. 2005, Porteus and Carroll 2005).

The application of ZFNs in plant genetic engineering has been successfully demonstrated in *Arabidopsis* (Lloyd et al. 2005), tobacco (Wright et al. 2005), and maize (Arnold et al. 2007). In tobacco protoplasts, the use of ZFNs greatly enhanced the frequency of localized recombination when measuring homologous recombination through restoration of a defective GUS::NPTII reporter gene. Gene targeting in the proper location was as high as 20% (Wright et al. 2005). In maize, multiple ZFNs were designed to target an herbicide resistance marker (PAT) to the ZmIPK1 gene, an endogenous maize gene encoding for inositol 1,3,4,5,6-pentakisphosphate 2-kinase, the enzyme responsible for the terminal step in phytic acid biosynthesis. PCR (polymerase chain reaction)-based genotyping and sequencing confirmed integration of the PAT cassette into the ZmIPK1 target (Arnold et al. 2007), though no integration fidelity and random transgene insertions in the genome were analyzed. These two studies are important confirmation that the use of ZFNs can overcome the hurdles of using homologous recombination for plant genome modification.

Researchers moved recently from working with small gene sequences (approximately 200 base pairs) to test the possibility of using ZFNs for “gene addition” or site-directed integration of long segments of DNA. Moehle and colleagues (2007) showed that ZFNs can direct the integration of an 8-kilobase (kb) sequence carrying three different transcriptional units into an endogenous locus of the human genome. This study demonstrates the versatility of ZFN technology for simultaneous gene targeting and gene stacking (Moehle et al. 2007). Although ZFN technology for gene targeting has been proven to work in several eukaryotic systems and presents the potential for numerous applications in genome remodeling, it is not yet widely used. One major limitation is the off-target DNA cleavage of ZFNs, which leads to cellular toxicity. Several groups have worked on redesigning the structure of the dimerization complex of the two ZFs in the ZFN to reduce the off-target cleavage frequency and cytotoxicity (Miller et al. 2007, Szczepek et al. 2007). Until recently, the design of highly specific ZFNs was difficult and required a considerable amount of time and input. Resources are now available for engineering ZFNs to be used in several systems (Wright et al. 2006).

Another strategy for overcoming the position effect and size restriction of transgenes is the development of plant artificial chromosomes for delivery of large DNA sequences, including large genes, multigene complexes, or even complete metabolic pathways (Preuss and Copenhagen 2006). In mammals and yeast (Houben and Schubert 2007), and more recently in plants (Yu et al. 2006, 2007, Carlson et al. 2007), artificial chromosomes have been produced by de novo construction using cloned components of chromosomes (Carlson et al. 2007) or through telomere-mediated truncation of endogenous chromosomes (Yu et al. 2006, 2007). Telomeres are composed of tandem motifs of highly conserved sequences found at the ends of chromosomes, where they play a protection role. By introducing telomeric sequences through *Agrobacterium*-mediated transformation or particle bombardment, it is possible to generate chromosomes with new telomeres, after generating a truncation at the end of the recipient chromosome. Yu and colleagues (2006, 2007) used a 2.6-kb telomeric sequence from *Arabidopsis* to transform immature zygotic embryos of maize. The telomeric sequence was flanked by sites for future manipulations in site-specific recombination such as the Cre/*lox* or FRT/*FLP* systems and by marker genes that could be visualized to detect the sites of integration and chromosomal truncation. Truncation of the maize chromosomes was demonstrated using fluorescent in situ hybridization karyotyping, showing expression of the marker genes at the chromosome ends (Yu et al. 2006). The utility of the site-specific recombination systems in the engineered chromosomes was also demonstrated (Yu et al. 2007). A transgenic plant line carrying the Cre recombinase controlled by a constitutive 35S promoter (35S-*lox66*-Cre) was crossed with another transgenic plant line carrying a red fluorescent protein marker gene with no promoter (*lox71*-DsRed). The cross yielded progeny plants that showed the expression of the DsRed gene, indicating the integration of the 35S promoter at the 5' end of the DsRed gene via precise recombination at the *lox* sites. This study exemplified the potential for using engineered chromosomes in crop species for site-directed integration and gene stacking. An added advantage of this system is that the engineered chromosome can be introduced or removed from a particular genotype through conventional breeding techniques.

More recently, the generation of *in vitro* assembled chromosomes was demonstrated in maize as well. Carlson and colleagues (2007) designed a minichromosome vector that

contained two marker genes and 7–190 kb of maize genomic sequences such as satellites, retroelements, and repeats found in centromeric regions. The vectors were delivered into maize embryogenic tissue, and the plants were recovered and analyzed for functionality of the maize minichromosomes, inheritance, and stability of the marker gene through four generations. The research group was able to demonstrate that a maize minichromosome can be an autonomous chromosome stably inherited through at least four generations, and can be maintained through meiosis and mitosis. Expectations are high for this new achievement, as it is a valuable tool for achieving gene stacking in corn, presumably reducing the amount of time required for the lengthy breeding programs that follow a single-trait transformation. Applications can also go beyond traditional agriculture: this technology allows the integration of metabolic pathways that can be used in the production of pharmaceuticals and industrial products in plants (Carlson et al. 2007).

Not all plants are created equal: Toward genotype independent transformation

One of the major impediments in plant genetic engineering is the fact that not all plant species are equally transformable. Even for a species that can be transformed by either *Agrobacterium* or biolistic gun, not all of the genotypes or varieties within that species are necessarily able to be transformed. For example, Nipponbare, a japonica rice variety, can be transformed readily by the *Agrobacterium* mediated method, but most indica rice varieties are recalcitrant to transformation regardless of the gene delivery method. The inability to transform any genotype at any time was one of the major drawbacks during the early development of GE crops. The transgene carrying the trait of interest has to be introgressed from a transformable laboratory genotype—often inferior in agronomic performance—into an elite genotype through hybridization and a lengthy breeding and selection program, followed by extensive evaluation to eliminate deleterious genomic background effects.

Great effort has been devoted to exploring and establishing tissue and cell cultures with transformation competency—the ability to integrate exogenous genes into the genome and the ability to regenerate to a fertile plant. With the exception of *Arabidopsis*, for which a nontissue culture-based approach to transformation is available, most plant transformation involves tissue culture processes via organogenesis or embryogenesis (Potrykus and

Spangenberg 1995, Wang 2006). Although research to fine-tune culture media and incubation conditions will continue to be important in improving transformation incrementally, more attention is now turning to understanding the molecular basis of plant regeneration and the interaction between plant and *Agrobacterium*. Nishimura and colleagues (2005) identified and isolated rice genes that are involved in regeneration. They found that a main quantitative trait loci gene encoding ferredoxin-nitrite reductase (NiR) determines regeneration ability in rice. The rice variety Koshihikari has poor tissue culture regeneration ability and a lower expression of the *NiR* gene compared with the highly regenerable variety Kasalath. When the Kasalath *NiR* gene was introduced into Koshihikari callus culture using the *Agrobacterium*-mediated method, the regenerability of Koshihikari was vastly improved (Nishimura et al. 2005). It was also found that stimulation of the cell cycle by disruption of the plant retinoblastoma pathway can lead to enhanced maize transformation frequency (Gordon-Kamm et al. 2002). When a replication-associated protein (RepA) from wheat dwarf virus was introduced into a tobacco suspension BY-2 cell culture by bombardment, significantly more cells were found to have undergone cell division. In maize, the transgenic maize plants carrying the RepA gene were observed to have greater embryogenic callus growth and could be retransformed with higher frequency.

We expect to gain further insights into better selection of target tissues and developmental stages for genetic transformation as more genomic information becomes available. Many groups have attempted nontissue culture-based methods for various plant species, inspired by the great success of the *Arabidopsis* floral dipping method. However, few reproducible protocols have been developed and reported to date. Although it is important to identify genes that may stimulate transformation or tissue culture responsiveness, transformation would be difficult if one first had to establish transgenic plants expressing the transformation-enhancing gene and introduce the gene of interest by the second transformation. Regardless of the method of choice, the most desired transformation technology should require the least input—labor, supplies, and growth space—and offer the highest efficiency and broadest spectrum.

In mother we trust: Plastid transformation

All deregulated commercial transgenic crops are, to date, nuclear transformants, with transgenes inserted into the nuclear genome of plants. In addition to transgene instability caused by position effects and complex transgene integration, nuclear transformation can also be of concern for undesired transgene dissemination through cross-pollination or pollen drift. This is especially important when pollen from transgenic plants is intended for nonfood production (e.g., pharmaceuticals or industrial enzymes). One of the proposed biological confinement measures is to express transgenes in the plastids, which are inherited through maternal tissues in most species (Bock 2007). Because plastid genomes are of prokaryotic origin, they can express several genes from single operons, a desirable characteristic for gene stacking. The plastid also has prokaryotic type gene expression machinery, which allows versatility in codon usage for recombinant protein production. A single cell in higher plants can contain thousands of copies of a single plastid gene, which for transgenes can result in high levels of protein expression—up to 45% of total soluble protein (Maliga 2004). Epigenetic interference with the stability of transgene expression is reduced in transplastomic plants (those whose plastid genome has been engineered), leading to reproducible and stable transgene expression.

Plastid transformation represents a major technological challenge to plant biotechnologists. Although chloroplast transformation has been achieved in several plant species such as tobacco, tomato, and soybeans (Bock 2007), only tobacco chloroplasts can be routinely transformed. Plant mitochondria, on the other hand, have never been transformed.

The difficulty of engineering the plastid genome lies in the large number (1000 to 10,000) of genome copies per cell distributed among numerous (10 to 100) plastids. Incorporation of the transgenes into the plastid genome through either homologous recombination or site-specific recombination is only the first step in obtaining a genetically stable plant, as it takes several cell generations and selection to dilute wild-type plastid genome copies. The use of spectinomycin and streptomycin resistance as a selective marker for plastid transformation has limited to some extent its application in cereal crops. Current cereal transformation protocols mostly use somatic embryogenesis processes that require

incubation in the dark, a condition under which spectinomycin becomes less effective. To date, no cereal chloroplast transformation has been reported (Maliga 2004, Bock 2007).

Even though plastid transformation is an attractive alternative for transgene containment, the technology still has several limitations that must be overcome before it can be widely used. It remains to be seen whether expression of a wide array of proteins that require certain posttranslational modifications, multimerization, or secretion is successful in the plastidial environment. Researchers should also focus efforts on deciphering the mechanisms of transgene expression in nonphotosynthetic plastids for their potential use in other tissues. Finally, control of transgene expression continues to be a major element in the success and safety of this technology.

Think small: Plant genetic transformation goes nano

Using nanoparticles for transmembrane delivery of DNA and drugs into animal cells is becoming a popular trend worldwide (Yih and Al-Fandi 2006). However, the fundamental differences between cell-wall-free animal cells and cell-wall-bearing plant cells present a major challenge for using these nano-particles in plant research. In the recently published first report on using nanoparticles for gene transformation in plants (Torney et al. 2007), it was demonstrated that mesoporous silica nanoparticles (MSNs), small surface functionalized silica particles, can be used to deliver both DNA and chemicals into either isolated plant mesophyll cells or intact plant leaves. The honeycomb-like nanoparticles can encapsulate chemicals in the pores, and their surface can be coated with DNA molecules. The most distinct advantage of this nanoparticle system over the current plant transformation methods is its ability to deliver more than one biological species to cells. Torney and colleagues (2007) loaded and capped a chemical (gene inducer or effector) inside the MSN, then coated the particles with DNA fragments carrying an inducible marker gene. Not only did they successfully deliver both chemicals and DNA into plant cells using the biolistic gun, they also were able to release the encapsulated chemical in a controlled manner to trigger the expression of the co-delivered transgene in the same cell.

The application of nanotechnology in plant transformation presents new possibilities for plant basic research and biotechnology. Because the MSN system has the ability to codeliver

more than one type of molecule, it provides opportunities to combine genes, hormones, enzymes, regulatory elements, RNA molecules, and chemicals in the study of gene function and cell development. For example, one may enhance the integration or recombination frequencies in plants by codelivering molecular components required for integration or recombination together with the targeted DNA fragments. Because the chemicals in the particle mesopores are encapsulated with covalently bound caps, they can, when necessary, be released by the introduction of uncapping triggers (chemicals that can cleave the covalent bonds attaching the caps to the MSN). This can be very useful if toxic compounds need to be delivered and released in a specific cellular compartment. Environmental changes (such as temperature or pH) could also be used as uncapping triggers to study the gene function and regulation at different plant developmental stages or environmental condition. Finally, it is possible to customize the MSN system by modifying the pore size and surface function of the nanoparticles. For example, functionalization with targeting sequences on the particle surfaces could allow the MSN and its contents to be delivered to specific cellular compartments.

From lab to field: Transgenics in action

Genetically engineered plants have played a pivotal role in the study of gene function and metabolic pathways. Historically, gene function in plants has been understood through the study of genetics as practiced by Gregor Mendel, or by using populations obtained through chemical and radiation mutagenesis. The T-DNA random insertion ability of *Agrobacterium* has been exploited to generate populations of transgenic plants carrying tagged T-DNA insertions for disruption of gene function and further biological study. Unlike mutation populations generated by chemical or radiation treatment, T-DNA tagged transgenic lines can readily be characterized at the molecular level. Using the known sequence of T-DNA, the site of insertion can be identified and the gene of interest can be cloned. The T-DNA tagged lines of multiple species are now available, including *Arabidopsis* (Galbiati et al. 2004, Ostergaard and Yanofsky 2004), rice (An et al. 2005, Hsing 2007), and barley (Ayliffe et al. 2007), among others. For studies on essential genes for which no viable mutants can be recovered, transgenic technology offers a tool for knockdown or knockin gene expression. Gene

functions of these essential genes can be studying by overexpressing them (knockin) or by down regulating or silencing them (knockdown), and observing the resulting phenotypes.

Perhaps the holy grail of breeders and biotechnologists is to be able to develop crops with significant improvements in yield. Transgenic approaches have been used for improving yield in the field by increasing biomass and biomass stability through crop protection, and more recently, by working on yield-enhancing genes. Crops have been engineered for traits such as sink strength, plant architecture, plant development, root growth, source strength, and assimilate partitioning (Van Camp 2005).

In crop protection, genetic engineering has provided the world with successfully commercialized insect-resistant and herbicide-tolerant crops, benefiting corn, soybean, and cotton growers around the globe (www.isaaa.org/resources/publications/briefs/35/highlights/default.html). As APHIS data on field release permit applications for 2007 show (Figure 2), efforts are ongoing to generate transgenic crops resistant or tolerant to biotic stresses such as viruses, fungi, bacteria, and insects (Castle et al. 2006). Abiotic stresses such as cold, drought, and salinity are also of major importance in agriculture, and numerous transgenic approaches have been taken to engineer tolerance in crops (Vinocur and Altman 2005).

Biotechnology has also made it easier to improve the nutritional quality of crops. Even though conventional breeding has been used successfully for this purpose since the beginning of agriculture, genetic engineering provides the added advantage of being able to supply traits or genes that are not present in available germplasm of some crops. Two landmark studies in this area are the engineering of the β carotene biosynthetic pathway in rice for enhanced provitamin A content (Ye et al. 2000) and the engineering of tomatoes for increased folate production (Diaz de la Garza et al. 2007). Interest is keen in using plant systems for “molecular pharming”—that is, using plants as biofactories for the production of recombinant proteins for pharmaceutical or industrial applications. This is largely due to the plant’s ability to produce a large quantity of “foreign protein” free of mammalian pathogen contamination. Over the last 10 years, molecular pharming has included production of proteins for pharmaceutical applications; enzymes, monoclonal antibodies, and antigens for vaccines or vaccine components; and other industrial products (Boehm 2007). Maize-derived

avidin, β glucuronidase, and trypsin have already gone through the regulatory process and are available commercially (Dunwell 1999, Horn et al. 2004, Boehm 2007). Recent interest in bioethanol has triggered interest in the production of cellulase enzymes in the plant system, which has been achieved with some success in maize (Biswas et al. 2006, Sticklen 2006). The study by Biswas and colleagues (2006) exemplifies the feasibility of producing heterologous cellulases in plants for biomass conversion to sugars and possibly biofuels.

Genetic engineering has been used in other areas for industrial and agronomic applications. For example, lignin content has been altered for use in paper pulping (Baucher et al. 2003) and for improved feedstock digestibility (Reddy et al. 2005). Polyhydroxyalkanoates, macromolecule polyesters considered ideal for replacing petroleum-derived plastics, have been produced in several plant species such as *Arabidopsis*, tobacco, maize, *Brassica napus*, alfalfa, flax, sugar beets, potato, and cotton (Suriyamongkol et al. 2007). Over the past decade, transgenic approaches have also focused on designing plants for phytoremediation (the use of plants for removing contaminants from the environment; Salt et al. 1998, Kramer 2005). Much interest has been shown in phyto-remediation of heavy metals from soil and water, contamination caused by mining, industry, agriculture and military practices. Transgenic plants capable of removing, containing, or sequestering cadmium, lead, mercury, arsenic, and selenium have been produced (Eapen and D'Souza 2005, Kramer 2005). New or improved varieties of floricultural crops have also been obtained by engineering traits such as color, shape, fragrance, vase life (in cut-flower species), rooting potential, and general plant morphology (Dunwell 1999, Casanova et al. 2005).

Concluding remarks

It is not an exaggeration to say that plant genetic engineering technology has changed the face of plant science, both in field practice in agriculture and in laboratory approaches in plant biology research. Despite concerns and skepticism, the first generation of commercialized transgenic crops has become the most rapidly adopted technology in modern agriculture. With rapidly expanding genomic information and improved transformation technologies, it is hoped that advances in biological research will bring a new generation of improved crop species to meet world demands for food, feed, fiber, and fuel.

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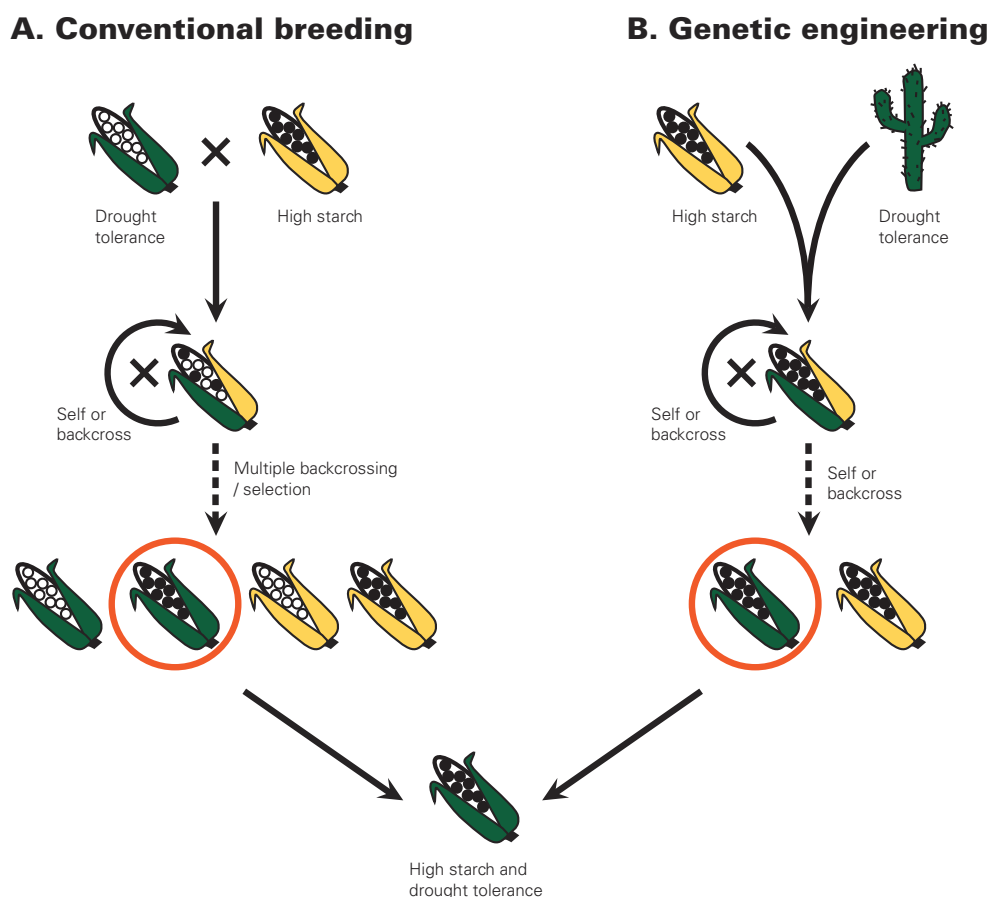


Figure 1. Plant improvement through conventional breeding and genetic engineering. Conventional breeding (A) identifies traits of interest in parent lines and incorporates them into a new variety through crosses (or hybridization). The resulting product can be further crossed to itself or backcrossed to another line of interest, thus further integrating the trait in the genetic background of choice. This approach, however, often results in a complex mix of genetic sequences integrated with the gene responsible for the desired trait. The process is lengthy and requires large amounts of seed and effort over several generations to achieve the final product. Crosses can be accomplished only between related species. Plant improvement through genetic engineering (B) allows the integration of genes conferring a trait of interest regardless of the donor species. For crop species, the procedure is tissue culture-dependent and often requires the backcrossing of the transgenic line to a line of desired agronomic performance before it can be released to the field. Though a complex task, genetic engineering allows crossing of the species barrier for trait utilization. Traits enhanced through genetic engineering are the same type as those selected for through conventional breeding, though genetic engineering has the added advantage of having no limit on the germplasm that can be used as donor species.

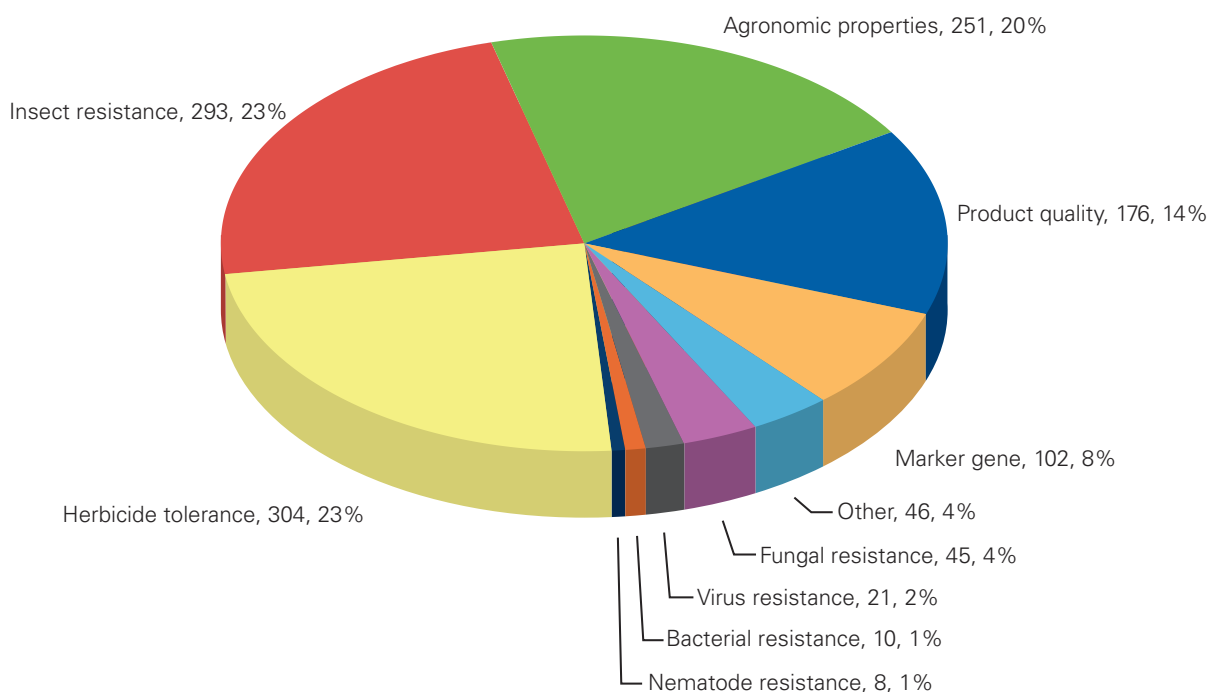


Figure 2. Permits approved by the Animal and Plant Health Inspection Service for field testing in 2007. A total of 671 release permits were issued for field-testing transgenic crops in the United States. The label for each category indicates the phenotype, the number of permits issued for that category, and the percentage it represents of the total number of traits tested. One permit can contain multiple phenotypes or phenotype categories. It is noteworthy that approximately 54% of traits pertain to crop protection through biotic stress resistance or tolerance. The phenotype category for agronomic properties includes traits such as resistance and tolerance to abiotic stress, altered amino acid composition, coloration, fertility, kernel development, lignin content, nitrogen metabolism, oil profile, floral development, and germination. The phenotype category “product quality” includes accelerated ripening, altered secondary metabolites, amino acid and oil levels, altered coloration, altered heat-stress response, altered kernel development, altered lignin biosynthesis, altered oxidative-stress response, altered cadmium metabolism, altered caffeine levels, improved digestibility, altered fiber properties, production of industrial enzymes, enhanced iron absorption, reduction of nicotine levels, reduction of phytate levels, and altered vitamin C content, among others. The phenotype category “others” includes antibody and antibiotic products, altered carbohydrate metabolism, anthocyanin levels, susceptibility to *Agrobacterium*, accumulation of fluorescent proteins, pharmaceutical protein production, protein tagging, and value-added protein production, among others. Details of phenotypic categories can be found at www.isb.vt.edu/cfdocs/isblists2.cfm?opt=28#AP.

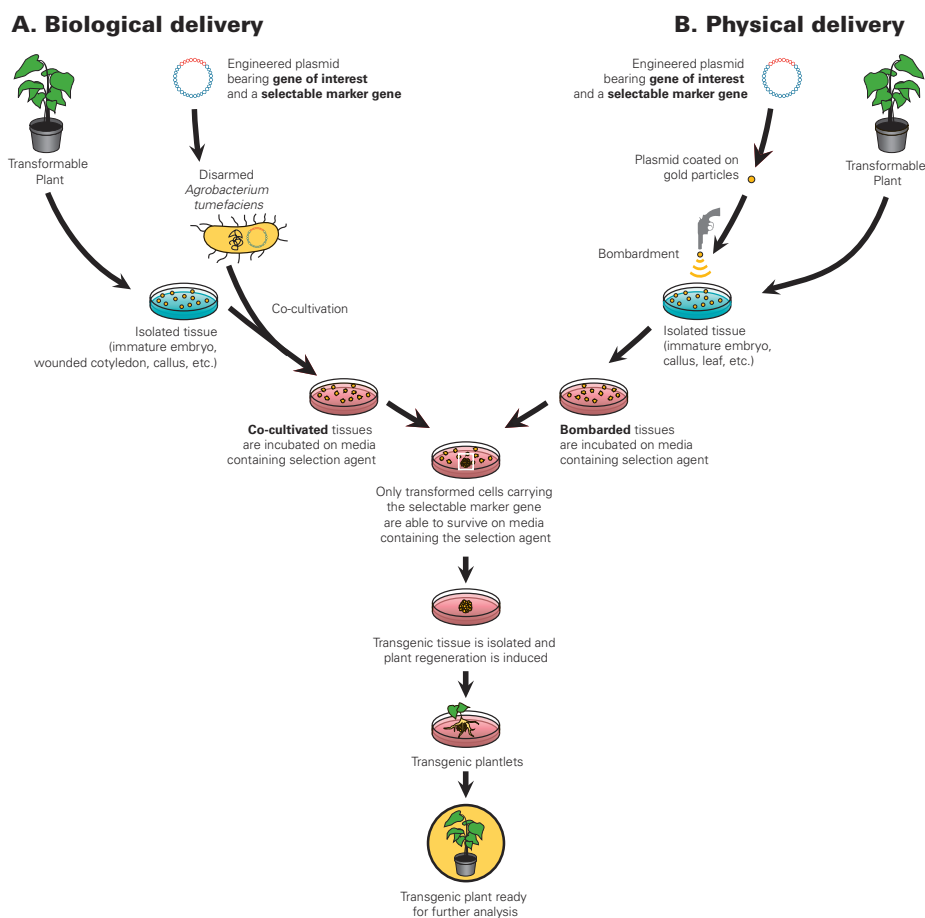


Figure 3. Two major approaches for the plant genetic transformation. (A) *Agrobacterium*-mediated transformation. This biological delivery system utilizes the natural DNA transfer ability of a soil bacterium, *Agrobacterium tumefaciens*. For plant transformation, researchers use a laboratory strain of *Agrobacterium* (i.e., disarmed strain) that has been engineered to remove its pathogenicity but retain its DNA transfer ability. The target plant tissue (e.g., leaf disc, immature embryos, callus material) is “infected” with the disarmed *Agrobacterium* carrying a plasmid that bears the gene of interest and an appropriate selectable marker gene (e.g., herbicide or antibiotic resistance). (B) Biolistic gun-mediated transformation. This is one of the most effective physical gene delivery systems for plants. Tungsten, gold, or silica nanoparticles are coated with a plasmid carrying the gene of interest and an appropriate selectable marker gene, and are bombarded into the tissue to be transformed. After delivery of the DNA via the *Agrobacterium*-mediated (A) or biolistic gun-mediated (B) method, the tissue is incubated on media containing nutrients and plant growth hormones for a short time before being taken to a similar medium that includes the phytotoxic selection agent (herbicide or antibiotics). Because of the presence of a selectable marker gene, the transformed cells can survive the media containing phytotoxins, whereas nontransformed cells will die. Herbicide or antibiotic-resistant tissue can be further cultured on media for regeneration to become a whole plant. These plantlets can be grown in the soil to maturity. Molecular and biological analysis of these plants can be conducted from this point forward.

CHAPTER 3: A BACTERIAL SIGNAL PEPTIDE IS FUNCTIONAL IN PLANTS AND DIRECTS PROTEINS TO THE SECRETORY PATHWAY

Lorena Moeller, Qinglei Gan and Kan Wang

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Abstract

The *Escherichia coli* heat labile enterotoxin B subunit (LT-B) has been used as a model antigen for the production of plant-derived, high-valued proteins. Previous work has shown that LT-B can accumulate in the starch granules of transgenic maize kernels. In the present study, the targeting properties of the bacterial LT-B protein and its native signal peptide (BSP) in plant systems were examined. A series of translational fusions of LT-B with the green fluorescent protein (GFP) at its C-terminus and various combinations of signal peptides at its N-terminus were generated and expressed transiently and stably in plant cells. Biochemical analysis of transgenic Arabidopsis and maize indicate that the LT-B::GFP fusion proteins can assemble and fold properly retaining both antigenicity of LT-B and fluorescing properties of GFP. The LT-B::GFP fusions and BSP-GFP strongly associate with both starch and fiber fractions of stably transformed maize seeds. Resistance to proteinase Thermolysin digestion of starch samples suggest the internalization of the fusion protein in the starch granules. Subcellular localization of the fusion proteins using confocal microscopy indicates that the proteins accumulate in the secretory system of Arabidopsis and maize in a signal peptide-dependent fashion. They also suggest that dual localization can be a species-specific event. The results provide important insights for further understanding the heterologous protein trafficking mechanisms and developing effective strategies for the production of recombinant proteins in plants.

Introduction

Production of recombinant proteins such as high valued pharmaceutical or industrial products in plants has many advantages, including safety and scalability of plant production systems (Streatfield, 2007). However, achieving high levels of recombinant proteins in a heterologous system is an ongoing challenge. Transgenic protein expression is driven mostly by the selection of strong and/or tissue specific promoters and specific targeting signals for proper compartmentalization within the cell. This allows for different levels of regulation and modifications that result in variable levels of recombinant protein expression and accumulation (Hood, 2004). The subcellular localization of a protein accounts greatly for its proper maturation and function within the cell, hence it affects the degree to which functional protein is accumulated.

Among the plant systems used for recombinant protein expression, high-yielding crops have received a lot of attention in the past decade. Maize has been demonstrated as an effective expression system for functional proteins of prokaryotic, viral and eukaryotic origins (Murry *et al.*, 1993; Hood *et al.*, 1997; Kusnadi *et al.*, 1998; Streatfield *et al.*, 2001; Streatfield *et al.*, 2002). The commercialization of corn-produced β -glucuronidase (Witcher *et al.*, 1998), aprotinin (Zhong *et al.*, 1999), and avidin (Hood *et al.*, 1997) demonstrated the viability of this expression system at the commercial level. Maize has also been used for production of edible vaccines against deadly diseases such as traveler's diarrhea through expression of the antigenic subunit B of *E. coli* heat labile enterotoxin (Chikwamba *et al.*, 2002a; Chikwamba *et al.*, 2002b; Streatfield *et al.*, 2003). Despite concerns over using grain crops for the production of recombinant proteins, both the scientific and economic advantages of maize-based approaches are undeniable (Ramessar *et al.*, 2008).

Maize seed is known for its large storage capacity and stability of proteins and starches, hence it is considered as an ideal organ for manufacturing recombinant proteins (Ramessar *et al.*, 2008). While there are extensive studies on maize native seed storage proteins (Shewry and Tatham, 1990; Shewry and Halford, 2002), knowledge related to heterologous protein production and storage are limited. A number of studies report the subcellular localization of the recombinant protein in the transgenic maize (Hood *et al.*, 1997; Witcher *et al.*, 1998) and the relationship between subcellular localization and transgenic protein yield (Streatfield *et*

al., 2003). These reports highlighted the importance of proper compartmentalization for optimum expression and accumulation of functional protein in plants. Expression of the *E. coli* heat labile enterotoxin subunit B (LT-B) gene in maize with its N-terminus native bacterial signal peptide or with a native 27 kD γ -zein signal peptide resulted in accumulation of LT-B in the starch granules of the transgenic kernels (Chikwamba *et al.*, 2003). This observation has raised a question on how bacterial protein and its signal peptide behave in the plant cellular machinery. Considerable interest has also arisen in the possible trafficking pathway of recombinant proteins in plants.

Protein translocation pathways in bacteria (Papanikou *et al.*, 2007; Driessen and Nouwen, 2008) and plants (Raikhel and Chrispeels, 2000; Hanton *et al.*, 2007; Hormann *et al.*, 2007; Brown and Baker, 2008; Rojo and Denecke, 2008) have been the focus of many studies and continue to be of great interest in today's research. In Gram-positive bacteria secreted proteins only need to cross the plasma membrane, while in Gram-negative bacteria, secreted proteins are translocated through the plasma membrane into the periplasm, and through the outer membrane. Most secreted proteins carry an N-terminal signal sequence that directs them to the inner membrane protein translocation machinery to be internalized into the membrane, translocated to the periplasm or secreted (Saier, 2006; Saier *et al.*, 2008).

In plants, however, the sorting of proteins in the cell is somewhat more complex due to the presence of different organelles, and hence, different types of membranes. Finely orchestrated gene regulation and tissue gene expression along with subcellular compartmentalization of plant proteins allows cells to become specialized and differentiated to fulfill their role in tissue specificity, organ identity and organism performance. Proteins carrying an N-terminal signal peptide are often directed to the secretory pathway, transported into the endoplasmic reticulum (ER) lumen, and sorted thereafter to the Golgi, cell wall, vacuole or plasma membrane, or retained in the ER. Proteins lacking a signal sequence are translated in the cytosol, and if carrying appropriate targeting signals, they are then translocated into other organelles such as the plastids (Inaba and Schnell, 2008; Paul, 2008) or mitochondria (Attardi and Schatz, 1988). More recently, two cases have been described in which proteins are targeted to the plastid through the secretory pathway (Villarejo *et al.*, 2005; Nanjo *et al.*, 2006). Two native plant proteins, α -carbonic anhydrase from

Arabidopsis (Villarejo *et al.*, 2005) and rice plastidial N-glycosylated nucleotide pyrophosphatase/phosphodiesterase (Nanjo *et al.*, 2006) were shown to localize to the plastids after being processed in the ER, describing a novel pathway for protein trafficking into the plastids.

Though much has been learned about the translocation machineries of bacteria and plants, less is known about how plants utilize their molecular translocation machineries to sort bacterial proteins when expressed in transgenic plants. LT-B has a native signal peptide that directs the protein for secretion in bacteria. In *E. coli*, LT-B is exported to the periplasm in a Sec – dependent fashion via the general secretory pathway. Once the signal peptide is cleaved, the protein assembles into a functional pentamer and associates with the A subunit (LT-A) to form the holotoxin before being secreted from the cell via a type II protein secretion pathway (Tauschek *et al.*, 2002). LT-B has been expressed in several plant species such as potato (Mason *et al.*, 1998; Tacket *et al.*, 1998), tobacco (Kang *et al.*, 2003), maize (Chikwamba *et al.*, 2002b; Streatfield *et al.*, 2003) and soybean (Moravec *et al.*, 2007). Though several of these studies describe the targeting of LT-B to different cellular compartments by the inclusion of targeting signals within their constructs (Streatfield *et al.*, 2003), only two studies show detailed subcellular localization of LT-B in the transgenic material (Chikwamba *et al.*, 2003; Moravec *et al.*, 2007). Streatfield and colleagues (Streatfield *et al.*, 2003), for example, used the signal sequences from barley alpha amylase and aleurin to target LT-B to the cell surface and vacuole, respectively, and the maize granule-bound glycogen synthase to target LT-B to the plastids. In the Chikwamba work (Chikwamba *et al.*, 2003) the functional LT-B was found in the starch granules of transgenic maize kernels expressing the LT-B with its native bacterial signal peptide. In the Moravec work (Moravec *et al.*, 2007) the replacement of bacterial signal peptide with an *Arabidopsis* basic chitinase signal peptide at the N-terminus of LT-B resulted in the localization of LT-B in protein bodies of transgenic soybean seeds.

The focus of this study is to examine the subcellular trafficking of the bacterial LT-B protein and its native signal peptide in plant systems using the green fluorescent protein (GFP) as a reporter. Using the functional LT-B::GFP fusion proteins it is shown that the LT-B signal peptide, not LT-B protein itself, can direct cargo proteins to the secretory pathway

in *Arabidopsis thaliana* and maize. Additionally, in maize seed the bacterial signal peptide leads to a strong association of the cargo proteins with the starch fraction, though most of the soluble fusion protein can also be found in the fiber fraction. The results provide insights for further understanding the processing of a bacterial protein in the plant cells and future design of a high level production system for recombinant proteins in plants.

Materials and methods

DNA constructs

A schematic representation of the constructs used in this study is presented in Fig. 1. The enhanced green fluorescence protein (EGFP) sequence in pLM01, pLM02, pLM03, pLM08 and pLM09 was cloned from p27zn-signal (Shepherd and Scott, 2009) using standard molecular biology techniques for restriction enzyme – based cloning. Construct pTH210 containing the CaMV 35S promoter (P35S), tobacco etch virus translational enhancer (TEV) and LT-B was used as a cloning vector (Mason *et al.*, 1998) for some of the constructs. For generation of plasmids pLM01, pLM02 and pLM03 the EGFP sequence was cloned into pTH210 at the NcoI-SacI sites, SacI-SacI sites, and KpnI-SacI sites, respectively. Plasmid pLM01 was used as a backbone for generation of pLM08 and pLM09. Plasmid pRC5, a pUC19-based vector carrying the maize 27kD γ -zein promoter (P γ zein), TEV, the maize 27kD γ -zein signal peptide (ZSP) fused to LT-B and the soybean vegetative storage protein terminator (Tvsp), was used as a source of LT-B fused to the maize 27 kD γ -zein signal peptide (Chikwamba, unpublished). The ZSP-LT-B fragment was amplified by PCR method and the digested product was cloned into the NcoI-BstXI site of pLM01. An NcoI-EcoRI fragment of pLM08 was inserted into the NcoI-EcoRI backbone of pLM01 to generate pLM09. A simple alanine-glycine linker (AG linker) consisting of six amino acids with three AG repeats (AGAGAG) was added between LT-B and GFP using oligonucleotide extensions in the PCR primers in plasmids pLM03, pLM08 and pLM09. Expression cassettes (Fig. 1) were cloned and recombined into a Gateway version of pTF101.1 (Paz *et al.*, 2004), pTF101.1gw1, for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plasmid pRC4 (Chikwamba *et al.*, 2002b) was used as a donor of the P γ zein, and derived plasmids.

The nuclear marker VirD2::RFP was a kind gift of Dr. Stanton Gelvin (Department of Biological Sciences, Purdue University, West Lafayette, IN, USA). The ER marker plasmid designated here as ER cherry (Nelson *et al.*, 2007) was obtained from the Arabidopsis Biological Resource Center (ABRC Stock # CD3-959, <http://arabidopsis.org>).

Stable transformation of Arabidopsis thaliana and maize

Transformation of *Arabidopsis thaliana* was done using standard protocols for floral dipping (Clough and Bent, 1998). Seed recovered from transformed plants was sown in flats and sprayed with Liberty 150 mg/L at 3, 6 and 9 days after germination. Resistant plants were taken to maturity and grown for image analysis.

Maize transformation was carried on at the Center for Plant Transformation at Iowa State University as described previously (Frame *et al.*, 2000). Briefly, DNA constructs (Fig. 1) were co-bombarded with a selectable marker gene that confers resistance to the herbicide bialaphos. Herbicide resistant calli were analyzed using the polymerase chain reaction (PCR) for presence of the GFP and LT-B genes. For constructs driven by P35S, the calli of transgenic events were tested for GFP fluorescence and imaged as described. Transgenic calli were regenerated and plants brought to maturity in the greenhouse.

Protein extraction from transgenic tissue

Endosperm powder samples were collected from transgenic kernels using a hand-held drill as described (Sangtong *et al.*, 2002). Plant materials were incubated with the following protein extraction buffer at a rate of 10 μ l buffer per milligram of maize powder: 25 mM sodium phosphate (pH 6.6), 100 mM NaCl, 0.1% Triton X-100 (v/v), 1 mM EDTA, 10 μ g/ml of leupeptin, 0.1 mM serine protease inhibitor Perfabloc SC (Fluka), for 2 hours at 37°C. Total aqueous extractable protein (TAEP) was determined using the Bradford assay (Bradford, 1976).

LT-B detection by G_{M1} capture ELISA

Quantitation of LT-B in the samples was carried on using a modification of the monosialoganglioside dependent enzyme linked immunosorbent assay (ELISA) described previously (Chikwamba *et al.*, 2003). The described protocol was modified as follows:

monosialoganglioside G_{M1} from bovine brain (G7641, Sigma, St Louis, MO, USA) was used at a 10 µg/mL concentration, 50 µL per well. Streptavidin-horseradish peroxidase conjugate (554066, BD Biosciences, San Jose, CA) was used at a dilution of 1:1000 in 1% dry milk (DM) (w/v) in phosphate buffered saline [PBS; 0.01 M Na₂HP0₄, 0.003 M KH₂PO₄, 0.1 M NaCl, (pH 7.2)]. Horseradish peroxidase substrate (ABTS; 0.5 mM 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt, 0.1 M citric acid, pH 4.35) was activated prior to use, by adding 5.5 µL 30% H₂O₂ to 5.5 mL ABTS solution. Activated horseradish peroxidase substrate was added to the plate and incubated in the dark at room temperature for 30 min. Absorbance was measured spectrophotometrically at 405 nm, at the end of the reaction. Sample wells were blanked against non-transgenic maize protein extracts and all measurements were performed in duplicate. Raw ELISA data was converted to percent LT-B of total soluble protein by reference to an ELISA standard curve constructed using purified bacterial LT-B (kindly provided by Dr. John Clements, Tulane University, LA, USA).

Western blotting

An aliquot containing 50–100 µg of total aqueous extractable protein (TAEP) from maize kernels were boiled for 5 min and loaded onto a 15% polyacrylamide SDS-PAGE (Laemmli, 1970). The separated proteins were transferred to a 0.45 µm nitrocellulose membrane using the BioRad Semidry Transblot apparatus according to the manufacturer's instructions. Membranes were blocked with 5% DM in PBST (PBS - 0.05% Tween-20 (v/v)) for 3 h at room temperature. Presence of GFP and LT-B::GFP fusions was evaluated by overnight incubation of the membranes in goat anti-GFP (Cat# 600-101-215, Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) diluted 1:8000 in 1% DM in PBST at room temperature. Rabbit anti-LT-B (RECO-55G, Immunology Consultants Laboratory, Inc., Newberg, OR, USA) was also used at a 1:1000 dilution in 1% DM in PBST and incubated for 1h at room temperature. Maize waxy and γ-zein proteins were probed using rabbit anti-waxy (1:2000; S. Wessler, University of Georgia, Athens, GA, USA) and rabbit anti-zein (1:3000; Dr. Paul Scott, USDA/ARS, Ames, IA, USA) antibodies, respectively. Membranes were washed four times with PBST. A horseradish peroxidase conjugated rabbit anti-goat

IgG (1:3000; A4174, Sigma, St Louis, MO, USA) or goat anti-rabbit (1:2000 for LT-B or 1:5000 for waxy and zeins; A0545, Sigma, St Louis, MO, USA) in PBST was used as secondary antibody. Colored bands were revealed by incubation with horseradish peroxidase substrate, 3,3',5,5'-Tetramethylbenzidine (T0565, Sigma, St. Louis, MO, USA).

Immunoprecipitation

For immuno precipitation, 500 µg of TAEP were incubated with 10 µL of rabbit anti-LT-B (RECO-55G, Immunology Consultants Laboratory, Inc., Newberg, OR, USA) overnight in the cold room (9-11°C). Protein G beads (IP50-1KT, Sigma, St Louis, MO, USA) were used to recover the immunoprecipitation (IP) complex, following suppliers recommendations. Ten µL of recovered IP complex was analyzed by western blotting using goat anti-GFP antibody as described in “Western blotting” section of the Materials and Methods.

Relative quantification of fluorescence in transgenic kernel protein extracts

Relative fluorescence was measured using a modified protocol described by (Shepherd and Scott, 2009). Briefly, 50 µL of TAEP from transgenic kernels were placed into a well of black, 96-well flat-bottom plate. The fluorescence of the extracts was measured at 485 nm excitation wavelength and 535 nm emission wavelength using a spectrofluorometer (Tecan, Mannedorf/Zurich, Switzerland). The plate was read in triplicate. Mean fluorescence levels were calculated for each sample after subtraction of blank fluorescence. Relative fluorescence was calculated as fluorescence per µg TAEP.

Small scale starch isolation from transgenic maize kernels

A modified protocol based on (Chikwamba *et al.*, 2003) was used for small scale starch isolation. Five to ten dry transgenic maize kernels were imbibed in sterile water at 37°C overnight. Alternatively, 5 to 10 transgenic kernels of ears harvested 15-20 d after pollination were directly dissected from the cob. The pericarp and embryo were removed from the kernels using a small bent forceps, and the dissected endosperms were placed in 10 mL of fresh sterile water in a conical 50 mL Falcon tube. The kernels were then homogenized using a Polytron homogenizer. Samples were then filtered using a 30 µm Nylon Filter (146506, Spectrum Laboratories, Houston, TX). The original tube was rinsed 3

times with water and all fractions were collected into a clean Falcon tube. The filtrate was centrifuged at 3000 rpm for 30 min, and the material on the filter was saved as the fiber fraction. After aspiration of the supernatant, the starch slurry was transferred to an eppendorf tube and centrifuged for 5 min at 5000 rpm. The starch pellet was washed three times each with water, 70% ethanol, 95% ethanol and 75% ethanol /3% mercaptoethanol, and then spun to dryness in a speed vac. The fiber was placed directly into a 50 mL falcon tube and lyophilized overnight. This fraction was designated as the fiber fraction.

Thermolysin treatment of starch samples

Thermolysin (3097-ZN, R&D Syetem, Inc., Minneapolis, MN, USA) was diluted to 10 $\mu\text{g/mL}$ with 5 mM CaCl_2 solution. Ten mg of starch were incubated with 100 μL thermolysin solution at 37°C for 2 h. The reactions were terminated by the addition of EDTA to final concentration of 20 mM. Samples were subsequently washed five times with 1 mL distilled water. After the last wash, 100 μL 1X SDS sample buffer was added to each sample and boiled 5 min. Twenty μL were loaded onto an SDS-PAGE gel for western blot analysis, as described in “Western blotting” section of the Materials and Methods.

Plant Material for transient assays

Arabidopsis thaliana (ecotype Columbia) seeds were vernalized in water at 4°C for 48 h before sowing in pre-wetted LC1 Sunshine Mix. Flats were placed in a growth chamber at 21°C and 16 h photoperiod covered with humidomes for 2-3 d. One to two weeks after germination, plants were thinned and transplanted to individual pots for further growth.

An *Arabidopsis thaliana* (ecotype Columbia) root culture was started from an ongoing culture in the laboratory of Dr. D. Bassham at Iowa State University, Ames, IA, USA. Cultured root cells were maintained as described (Contento *et al.*, 2005) using Lindsmaier-Skoog medium (LSP003, Caisson Laboratories, North Logan, UT) supplemented with 20 g/L sucrose, 1 mg/L naphthalenacetic acid (NAA) and 0.05 mg/L kinetin.

***Arabidopsis thaliana* mesophyll and root culture protoplast isolation and transformation**

Isolation of mesophyll protoplasts was carried out as described (Sheen, 2001). For isolation of root culture protoplasts, 10 to 20 mL of root cell culture were pelleted in a 50 mL Falcon tube by centrifuging 4 min at 50 x g, or resting on the bench. The supernatant was removed and replaced by 40 mL of fresh enzyme solution (0.5x artificial sea water [ASW, 1.7 M NaCl, 9.4 mM MgSO₄, 3.4 mM CaCl₂, 5 mM MES, 3.45 mM KCl, 8.35 mM MgCl₂.6H₂O, 0.875 mM NaHCO₃, pH 6.0], 0.3 M mannitol, 0.31% Cellulase Onozuka R10, 0.15% Macerozyme R10, pH 5.7, filter sterilized). Cells were transferred to a deep Petri dish and were vacuum infiltrated for 5 min. The petri plate was covered with aluminum foil and placed on an orbital shaker for 3.5 h at 50 rpm. The digested cell solution was filtered through a 40 µm mesh into a 50 mL conical Falcon tube, and the plate was washed once with cold W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl and 2 mM MES, pH 5.7, filter sterilized). Cells were spun at 50 x g for 4 min. The supernatant was removed and cells were washed twice with W5. The cells were carefully resuspended in fresh W5 and incubated on ice for at least 30 min prior to transformation with polyethylene glycol (PEG). Leaf and root protoplasts were transformed using PEG using the protocol described in (Sheen, 2001).

Sample preparation for microscopy

Mesophyll and root culture protoplasts of *Arabidopsis thaliana* were washed and resuspended in fresh W5 media. Thirty µL of protoplast solution were placed directly on the microscope slides for visualization. Transgenic *Arabidopsis thaliana* seeds were selected in ½ strength MS medium supplemented with 5 mg/L of bialaphos. Seedlings 4–7 d after germination were placed directly on a microscope slide and mounted using Vecta Shield (H1000, Vector Laboratories, Burlingame, CA, USA) mounting media.

Transgenic maize callus and developing endosperm were mounted directly onto microscope slides using Vecta Shield mounting media with propidium iodide (H1200, Vector Laboratories, Burlingame, CA, USA).

Fluorescence imaging and laser scanning confocal microscopy

Protoplasts, stable transgenic *Arabidopsis* seedlings, maize callus and maize endosperm samples were visualized using a Leica TCS/NT (Leica Microsystems Inc., Exton, PA, USA) laser scanning confocal microscope equipped with Argon (488 nm) and Krypton (568 nm) lasers and a double dichroic DD488/568 filter. Green fluorescence was detected using a combination of RSP580 and BP525/25 filters under wavelength between 500 and 550 nm. Red fluorescence was detected using a LP590 filter. During scanning the pinhole was maintained at 1 (airy units) for all images. Images were processed and analyzed using ImageJ software (Abramoff *et al.*, 2004).

Whole plants (*Arabidopsis*) and ears from self-pollinated transgenic maize plants harvested at 10–14 days after pollination were visualized and imaged using an Olympus SHZ10 stereoscope (Leeds Precision Instruments, Inc., Minneapolis, MN, USA) coupled to a SPOT RT color CCD camera (Diagnostic Instrument Inc., Sterling Heights, MI, USA). Images were taken under bright field or using a band pass exciter at 460-490 nm with emission filter 510-550 nm (for GFP detection), and acquired using SPOT Advanced software.

Results and Discussion

Protein fusions of heat labile enterotoxin subunit B (LT-B) and the green fluorescent protein (GFP) assemble and fold properly in plant cells

Constructs listed in Fig. 1 were made to investigate the subcellular targeting ability of LT-B signal peptide and LT-B protein itself using GFP as marker for visualization. The first two constructs have GFP fused to either no signal peptide (Fig. 1A, control), or the bacterial signal peptide (BSP) of LT-B (Fig. 1B). The last three constructs have the GFP fused to the C-terminus of the full length LT-B protein with the native BSP (Fig. 1C), a maize 27 kD γ -zein signal peptide (ZSP) (Fig. 1D), or no signal peptide (Fig. 1E).

To facilitate rapid construct verification and protein localization study in different plants, each construct set was made with two different promoters. A constitutive CaMV35S promoter was used for transient and stable analysis in *Arabidopsis* and maize callus culture,

while a maize seed specific 27 kD γ -zein promoter was used for endosperm analysis in transgenic kernels. For each construct, a transgenic line identification was designated and multiple independent transgenic events were generated (Fig. 1). For transgenic Arabidopsis, the number of stable transgenic events is based on herbicide selection on T₁ seed and confirmed by visualizing the T₂ seedlings under the fluorescence stereomicroscope. For maize, all transgenic lines were confirmed by gene specific PCR analysis of transgenic callus. Their transgene insertion copy numbers were estimated by Southern blot analysis (data not shown). Strong transgene expressing events were chosen for further localization and biochemical analyses.

To examine whether LT-B and GFP retain their functional properties in the fusions described, experiments were carried out to assess their correct folding and assembly in Arabidopsis and maize. In stable transgenic Arabidopsis, green fluorescent seedlings could be easily distinguished when the fusion constructs were present (data not shown). Similarly, it was possible to detect green fluorescent transgenic maize seeds (Fig. 2A), indicating that GFP, a molecule capable of folding without the need of chaperones, is active and correctly folded.

A monosialoganglioside (G_{M1}) capture Enzyme Linked Immunosorbent Assay (ELISA) was used to determine whether the LT-B protein assembles into functional pentamers when fused to GFP protein. To be biologically functional, the 11 kD LT-B protein needs to be correctly assembled into pentameric form, which can then bind to monosialoganglioside specifically (Spangler, 1992). Fig. 2B shows that multiple events of transgenic maize lines P310 and P311 accumulate pentameric LT-B at various levels.

These results combined indicate that both LT-B and GFP were able to retain their properties when fused; LT-B being in a pentameric form remains recognizable by its receptor, the G_{M1} receptor, and GFP retains its characteristic of self catalysis for folding and fluorescing.

Western blot analysis using anti-GFP and anti-LT-B antibodies on total aqueous extractable protein (TAEP) (Fig. 2C and 2D) were conducted to further confirm the presence of the fusion proteins in transgenic maize kernels. As shown in Fig. 2C, using anti-GFP antibody it was possible to detect bands around 27 kD (arrowheads) in transgenic lines P309

(P γ zein-GFP control) and P315 (P γ zein-BSP-GFP) as predicted. In P315, two bands with close molecular weights likely represent populations of unprocessed BSP::GFP and processed GFP proteins in the cells. On the other hand, in transgenic maize lines P308c (P35S-BSP-LT-B::GFP), P310-26 and -32 (P γ zein-BSP-LT-B::GFP), and P311 (P γ zein-ZSP-LT-B::GFP), the analysis also detects a number of bands that cross-reacted to anti-GFP antibody. One high-molecular-weight band estimated around 39 kD (asterisks in Fig. 2C) is likely the LT-B (11.7 kD) and GFP (26.9 kD) fusion product. Bands at the lower molecular weights (dots in Fig. 2C) cross-reacting to the antibody suggest possible proteolytic degradation of the fusion protein in these transgenic lines.

The anti-LT-B antibody (Fig. 2D) cross reacted with bands around 39 kD in P308c and P310 (asterisks in Fig. 2D), as well as bands around 11 kD (diamonds in Fig. 2D) in bacterial LT-B standard and P77, a control transgenic maize line expressing LT-B fused to its native signal peptide in the endosperm (Chikwamba *et al.*, 2002b). The 39 kD bands are likely the LT-B::GFP fusion proteins, while the 11 kD bands are proteolytic products that cross react with the anti-LT-B antibody. No LT-B cross-reacting bands are seen in constructs carrying GFP alone (P309 and P315), as is expected.

It is noteworthy that the ratio of fusion protein to degraded protein is markedly different between line P308c (P35S-BSP-LT-B::GFP) and P310 (P γ zein-BSP-LT-B::GFP) in both western blots using either GFP or LT-B antibody (Fig 2C & D). In both cases, a higher ratio of fused protein is detected in line P310 than in line P308c. When LT-B antibody was used, very little degraded bands were detected in P310 (Fig. 2D) at all. This observation is intriguing because the main difference between the two constructs is the promoter; P308c has the constitutive P35S promoter and P311 has the seed-specific γ zein promoter. As the consequence of promoter specificity, the TAEP of P308c was extracted from the stably transformed callus culture while the TAEP of P310 was from endosperm of transgenic seed. Because only limited numbers of callus and seed lines were analyzed, it was unfeasible to determine whether this difference was due to different fusion protein processing in the different tissues expressed or due to the protein extraction process itself.

Fig. 2E shows an anti-GFP western blot on immunoprecipitated transgenic maize kernel samples using anti-LT-B antibody. A reacting band was detected around 39 kD position in

maize lines P310 and P311, but no band could be seen in two control maize lines P309 (Pyzein-GFP) and P77 (Pyzein-LT-B, Chikwamba *et al.*, 2002b) carrying GFP or LT-B alone, respectively. This result confirms that the ~39 kD bands observed in Fig. 2C are, in fact, fused to LT-B, as predicted.

These data indicate that the C-terminal fusion of LT-B with GFP (using a simple alanine-glycine linker) results in retention of properties and assembly for both proteins. Because the pentameric LT-B protein is a potent antigen (Nashar *et al.*, 1998) and binds specifically to G_{M1} receptor of epithelial cell surface, our results presented here suggest that LT-B has potential to be used as a carrier molecule to deliver proteins larger than itself.

LT-B::GFP fusion protein is detected in starch and fiber fractions of transgenic maize kernels

Small scale starch isolation of maize kernels using a modification of the previously described protocol (Chikwamba *et al.*, 2003) gives two main fractions: starch and fiber. As in other wet milling fractionation procedures (Johnson, 2000), this protocol allows separation and recovery of starch, fiber, germ, steep water and pericarp. For practical purposes in this study, pericarp, embryo and steep water are discarded, and the endosperm is used for further separation of starch and fiber. While the starch fraction derives mostly from the amyloplasts, less is known about the cellular components of the fiber fraction.

Both starch and fiber fractions were analyzed for functional GFP and LT-B content in transgenic lines P310 (Pyzein-BSP-LT-B::GFP) and P311 (Pyzein-ZSP-LT-B::GFP). For a GFP expression assay, arbitrary fluorescence units of total aqueous extractable protein (TAEP) was measured using a protocol modified from (Shepherd and Scott, 2009). The TAEP of all samples were obtained by two different extraction methods: simple vortex at room temperature (25°C) and shaker-incubation at 37°C for two hours. These two methods of protein extraction were chosen to investigate the stability of the fusion protein by the previously described method of LT-B extraction (2 h/37°C, Chikwamba *et al.*, 2002b; Chikwamba *et al.*, 2003) compared to a more conservative extraction protocol (vortex/RT). As shown in Fig. 3A, fluorescence readings of TAEP obtained from 2 h/37°C incubation were generally higher than those from vortex/RT extraction, suggesting a better soluble

protein recovery was achieved when using a method with higher temperature and longer incubation time. Both extraction methods showed that stronger fluorescence was detected in the fiber fractions than in the starch fractions, for the LT-B::GFP fusion constructs (P310 and P311) as well as GFP alone constructs (P309 and P315).

The fraction association of the fusion proteins was also observed for LT-B protein (Fig. 3B). Functional pentameric LT-B in transgenic starch or fiber fractions expressing LT-B::GFP fusion led by either the BSP (P310) and ZSP (P311) was quantified using G_{M1} specific ELISA. The data in Figs. 3A and 3B indicate that LT-B is not only associated with the starch fraction as was previously reported, but also associated, at a higher percentage, with the fiber fraction obtained using the small scale fractionation protocol. This association was also observed for LT-B transgenic maize line P77 (Pyzein-BSP-LT-B; data not shown), the original line used in the previous work to demonstrate the LT-B association to the starch granules of transgenic maize kernels (Chikwamba *et al.*, 2003). The association of LT-B to fiber was not reported in the previous work was due to the fact that protein recovery from non-soluble and non-starch fractions was not determined. The observation that the LT-B has great association with the fiber fraction may provide a unique opportunity for effective downstream processing of a potential edible vaccine product, adding usefulness to the low value fiber fraction that results from corn fractionation (L. Johnson, personal communication: Center for Crops Utilization Research, Iowa State University, Ames, IA, USA).

Western analysis using the anti-GFP antibody on proteins extracted from aqueous phase and insoluble pellet phase of each fraction (Fig. 3C & D) were performed to confirm the presence of the fusion proteins in starch and fiber. Interestingly, strong fusion protein bands were observed in P310 and P311 in both starch pellet (asterisks in Fig. 3C) and fiber pellet (asterisks in Fig. 3D). Weak but detectable fusion protein bands can be seen in the fiber TAEP of P310 and P311 (asterisks in Fig. 3D). However, while LT-B was detectable by G_{M1} capture ELISA specific for LT-B detection, no protein bands were observed from the starch TAEP for both P310 and P311 (Fig. 3C). As for the control construct, the GFP protein from line P315 (Pyzein-BSP-GFP) behaved similar to P310 (Pyzein-BSP-LT-B::GFP) in the Western analysis. No detectable GFP band was seen for P309 (Pyzein-GFP), which carries the GFP with no signal peptide.

Thermolysin (EC 3.4.24.27) treatment based on published protocol (Chikwamba *et al.*, 2003) was used to verify whether the detection of the GFP and its fusion protein in starch pellets was due to internalization in starch grains of the insoluble phase as observed previously for LT-B alone (Chikwamba *et al.*, 2003). Any polypeptides within the starch granules should not be susceptible to hydrolysis upon treatment of intact granules with exogenous proteases such as Thermolysin (Mu-Forster and Wasserman, 1998). Starch pellets from three transgenic lines, two carrying GFP constructs (P309 and P315) and one carrying LT-B::GFP fusion construct (P310), were treated with Thermolysin. The fiber fraction of P315 was used as a treatment control. Both treated and untreated samples were subjected to Western blots that cross reacted with anti-GFP, anti-waxy and anti- γ -zein antibodies. If the proteins are internalized in the starch granules, the reacting band should remain the same intensity in Thermolysin treated and untreated samples. As can be seen in Fig. 4A, the GFP band in the Thermolysin-treated-control sample P315F (fiber fraction) was reduced. However, the P310 fusion protein band (asterisks in Fig. 4A) and P315 GFP band (arrowheads in Fig. 4A) in the Thermolysin treated and untreated starch samples remained same intensity, suggesting that these proteins were not sensitive to Thermolysin treatment, and most likely are protected by the starch granule membrane.

To monitor the Thermolysin effectiveness, maize waxy protein (Mu-Forster *et al.*, 1996) was used as an internal control for starch bound proteins and 27 kD γ -zein (Mu-Forster and Wasserman, 1998) was used as a marker for starch granular external protein contamination. Because the waxy protein is strongly associated with the starch granule, Thermolysin treatment did not reduce its protein band intensity in all samples (block arrow in Fig. 4B). On the other hand, the band intensity of the 16 kD and 27 kD γ -zeins (open block arrow in Fig. 4C), known to accumulate in protein bodies and not inside of starch granules, was notably reduced upon treatment with the protease (Fig. 4C).

The starch localization property of BSP signal peptide-led proteins is in complete agreement with previous observations in which the LT-B with its native BSP was found inside of starch granules of transgenic maize (Chikwamba *et al.*, 2003). This study provides further evidence that the presence of BSP or ZSP signal peptide could lead the cargo proteins to both starch and fiber fractions. On the other hand, when GFP is expressed in maize seed

without any signal peptide (P309), no GFP bands could be detected in starch fraction (Fig. 3C).

The bacterial signal peptide of LT-B is sufficient for localization of GFP and LT-B::GFP fusions to the secretory system of Arabidopsis and maize

Confocal microscopy was used for localization studies in transiently and stably transformed *Arabidopsis* cells, and stably transformed maize callus and endosperm tissue using the constructs described in Fig. 1 with the purpose of establishing the subcellular targeting properties of the bacterial signal peptide of LT-B and the LT-B protein itself. Figure 5 summarizes the results.

Figure 5A is the GFP control construct in which the *gfp* gene is under control of either P35S or Pyzein promoters with no signal peptide. The transient expression of GFP in *Arabidopsis* leaf (Fig. 5a) protoplasts, root (Fig. 5b) protoplasts, and seedlings (Fig. 5c), and stable expression of GFP in maize callus (Fig. 5d) and endosperm (Fig. 5e) resulted in localization of GFP in the cytoplasm and nucleus. This observation has been reported and reviewed extensively (Hanson and Kohler, 2001; Berg *et al.*, 2008).

The fusion of GFP to the C-terminus of the LT-B signal peptide BSP (Fig. 5B) resulted in localization of the GFP signal in the endoplasmic reticulum (ER) and ER-derived bodies in all types of transformed cells tested in this study (Fig. 5f–j). In *E. coli* enterotoxigenic strains, BSP is the signal peptide that directs LT-B protein to be translocated to the periplasm where the BSP is cleaved off, and the LT-B protein assembles into functional pentamers before being secreted in a folded state (Tauschek *et al.*, 2002). In most eukaryotic systems, and specifically in plants, secreted proteins are typically processed in the ER (Vitale and Boston, 2008). Two bands with similar molecular weights were detected (Fig. 2C) in western blotting in maize seed expressing the BSP::GFP construct (P315). On the contrary, only one GFP band was seen from seeds expressing the control GFP construct with no signal peptide (P309, Fig. 2C). It is likely the two closely migrating bands from P315 represent two populations of unprocessed BSP::GFP and processed GFP proteins with BSP cleaved.

To understand whether the mature LT-B protein also plays a role in subcellular localization in plant cells, the LT-B::GFP fusion construct linked with either bacterial BSP

(Fig. 5C), plant ZSP (Fig. 5D) or no signal peptide (Fig. 5E) was assessed in various transiently or stably transformed plant cells. The results show that the GFP signals were accumulated in the secretory systems for both BSP (Figs. k–o) or ZSP (Figs. p–t) led LT-B::GFP fusion proteins in both *Arabidopsis* and maize, similar to the observations presented Figs. 5f–j. On the other hand, when the signal peptide (Fig. 5E) was removed, the GFP signal returned to the cytosol and nucleus, and no signal was observed in the secretory pathway (Figs. 5u–w).

Figure 6A–G shows the co-localization experiments for the confirmation of subcellular localization using a known ER marker (Nelson *et al.*, 2007). This marker (ER cherry) contains a red fluorescent protein fused to the signal peptide of the *Arabidopsis thaliana* wall-associated kinase 2 (He *et al.*, 1999). *Arabidopsis* root protoplasts were co-transformed with ER cherry marker construct and various constructs listed in Figure 1. The results show that the ER-targeted RFP signals co-localized with the GFP signals from the constructs that contained either BSP (Figs. 6B & C) or ZSP (Fig. 6D). GFP signals from constructs carrying GFP alone (Fig. 6A) or LT-B::GFP fusion (Fig. 6E) with no signal peptide were constantly detected in separate subcellular compartments than ER-targeted RFP signal.

These combined results show that BSP plays the role of a signal peptide in plants, and results in localization of the fusion proteins in the secretory pathway of *Arabidopsis* and maize. It is also clear that the mature LT-B protein has no targeting ability for secretory system, and that the signal peptide alone, is sufficient for targeting cargo proteins to the ER.

The observation of the ER localization properties of LT-B signal peptide may explain why a great proportion of LT-B can be detected in the fiber fraction during the kernel fractionation. The fiber fraction generated in the small scale starch preparation process contains most maize cell components except for the starch, after removal of germ and pericarp by manual excision. The GFP and LT-B::GFP fused with BSP were found in both starch and fiber fractions, but GFP alone with no signal peptide was not detected in the starch fractions.

The results do not explain, however, why a fraction of GFP and LT-B::GFP proteins fused to BSP was also found in the starch granules of maize seeds. Starch granules are inside of the amyloplasts, the specialized storage plastids of maize endosperm. In plant cells

proteins targeted to the ER carry a signal peptide at their N-terminus that allows the recognition of the propeptide and its insertion and translocation into the ER lumen. The ER is the point of entry for the secretory pathway, which includes the ER, the Golgi, the plasma membrane, the vacuole, the cell wall or any body derived from any of these (Robinson *et al.*, 2007). On the other hand, nuclear proteins targeted to the plastids usually are translated on free ribosomes in the cytosol, and then are translocated into the plastids using the organelle's specialized import machinery in a transit peptide-dependent fashion.

Recently, two groups described a novel pathway for protein trafficking into the plastids. Accumulation in chloroplast stroma of α -carbonic anhydrase (α -CA), a protein predicted to go to the secretory pathway, prompted Villarejo *et al.* (2005) to further study the mechanism by which this protein reached the plastids. A series of biochemical tests, import assays, confocal microscopy and inhibition studies confirmed that α -CA is targeted to the ER and further processed within the secretory pathway before being transported to the chloroplast. Another exemplification of the existence of a pathway for protein trafficking of glycosylated proteins from the ER–Golgi system to the plastids in plants came from detailed study of the nucleotide pyrophosphatase/phosphodiesterase (NPP) of rice and barley (Nanjo *et al.*, 2006). A combination of biochemical and immunocytochemical analyses and confocal fluorescence microscopy of rice cells expressing NPP1::GFP fusions revealed that NPP1 is glycosylated and its accumulation in the plastids is dependent on a vesicular transport pathway from the ER and Golgi.

In an attempt to use some of the described tools, the glycosylation status of GFP and LT-B::GFP proteins in transgenic maize seeds was examined. LT-B protein carries one glycosylation consensus sequence (Asp-Lys-Thr) but is not predicted to be glycosylated (NetNGlyc 1.0), and the GFP used in this work does not have a glycosylation sequon at all. Preliminary analysis using Pro-Q Emerald green stain did not reveal any glycosylated bands for the molecular weights of the GFP, LT-B or LT-B::GFP fusions (data not shown).

The BSP-dependent dual localization observed in this work has some similarity to plant signal peptide-dependent dual localization reported for rice amylases (Chen *et al.*, 2004; Asatsuma *et al.*, 2005). These studies show the dual localization of rice α -amylase, α -Amy3 (Chen *et al.*, 2004), and α -amylase I-1, α -AmyI-1 (Asatsuma *et al.*, 2005), to the plastids and

cell wall/extracellular space in plant cells. For both studies, the dual localization was dependent on the presence of the native plant signal peptide. The detection of glycosylated forms of α -AmyI-1 in both soluble and chloroplast fractions led the authors conclude that this protein follows a route from the ER to the plastids by a novel mechanism as well (Asatsuma *et al.*, 2005). It remains to be determined whether the dual localization property of LT-B involves one or more trafficking routes, reaching the plastids via an ER-independent pathway.

A second explanation for the observations of the present work is that the default localization of the BSP-driven proteins is the ER as was observed in Arabidopsis and maize, but alternate fates occur in maize endosperm. Unexpected patterns of recombinant protein deposition in endosperm have been reported for other species such as wheat (Arcalis *et al.*, 2004) and rice (Drakakaki *et al.*, 2006). In wheat, KDEL-tagged recombinant serum albumin was shown to accumulate in ER lumen in leaves but was detected in prolamin aggregates inside of vacuoles in the endosperm, in a similar fashion as what was observed with recombinant phytase targeted for secretion (Arcalis *et al.*, 2004). In the same wheat study, recombinant legumin targeted to the vacuole resulted in accumulation of the protein in globulin inclusion bodies around the prolamin bodies (Arcalis *et al.*, 2004). Further effect of tissue type on recombinant protein subcellular fate was studied in rice by the expression in leaves and endosperm of recombinant phytase targeted for secretion. While in leaves the phytase was successfully secreted, in endosperm it was retained in the ER-derived protein bodies and protein storage vacuoles (Drakakaki *et al.*, 2006).

These examples and the observations presented for BSP-driven proteins suggest that intracellular fate of fusion proteins could be due to the nature of the tissue and cell arrangement in maize endosperm, with the possibility of functional proteins reaching the plastid via the secretory system. This hypothesis seems to be supported by two observations in this study. First, no GFP localization was observed in plastids of Arabidopsis protoplasts or leaf tissues in either transient or stable experiments. Second, LT-B::GFP protein fused to a native plant signal, ZSP (γ -zein protein signal peptide), was detected in starch granules of maize seed as well. In maize, the native γ -zein proteins are retained in the ER-lumen and accumulate in ER-derived protein bodies as a result of protein-protein interactions with other

zeins and chaperones (Kim *et al.*, 2002). Unlike the zein protein, the LT-B::GFP mature protein may or may not have the ability to interact with other zeins and ER-resident proteins to be retained effectively in the ER or its derived systems. A portion of the LT-B::GFP fusion protein would continue its path in the secretory pathway, and potentially take a route to the plastids through a mechanism that has yet to be described for a bacterial protein.

Expression of recombinant proteins in plants continues to be an area of great interest in the pharmaceutical, industrial and vaccine industries. The production of recombinant proteins in crop systems, in particular, represent a potential means for commercial scale production of proteins of interest in the future due to the attractive features of scalability, safety and stability. However, the process of going from a laboratory experiment to a large scale production can take years due to the lengthy and elaborate regulatory process and public reluctance for accepting the product. Many of the consumers' fears are due to lack of information on the system used for the recombinant protein production, and on how the heterologous proteins behave in the plants. Though many studies have investigated and documented the production of recombinant proteins in plants, very few papers have focused on the basic understanding of recombinant protein traffic within the cell.

In this study, the subcellular localization of the bacterial signal peptide of LT-B (BSP) and the LT-B protein using GFP as a visual marker was examined. Results show that the BSP, a bacterial signal peptide, behaves as such in *Arabidopsis* and maize, and is capable of delivering proteins to the secretory system in plant cells. Data also show that a successful fusion can be accomplished between LT-B and a large protein, GFP, and that both proteins retain their functionality when expressed in plant cells. Though the use of LT-B as a carrier has been extensively studied in bacterial systems, reports of plant-derived LT-B fusions are few. Two studies report the fusion of small molecules (5 – 6 kD) to the carboxy terminus of LT-B (Rigano *et al.*, 2004; Rosales-Mendoza *et al.*, 2009) . In both of these cases, the peptides used in the fusion represent about one-fifth of the size of the GFP protein (27 kD) used in this study. The results presented here are an important contribution to the oral vaccine research in that the margin of size allowed for fusions with LT-B has been extended far beyond what originally was thought possible, especially since it has been proven that both proteins retain their native conformations and functionalities. Because pentameric LT-B is

antigenic, it may be possible to design future vaccine strategies by linking LT-B with non-antigenic peptides and proteins to improve their efficacy.

Successful recombinant protein expression in plants usually requires a combination of factors including selection of the gene of interest, selection of regulatory elements and selection of targeting signals for proper compartmentalization within the cell. For many recombinant proteins of bacterial origin, researchers simply remove the native signal sequence (if any) and replace it with the plant targeting sequence of interest. In this study we present a detailed study of the role of a bacterial signal peptide in the localization of proteins in plants. To our knowledge, this is the only bacterial signal peptide that is shown to act as a plant signal peptide directing proteins to the secretory pathway in *Arabidopsis* and maize.

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Figure legends

Figure 1. Gene cassettes, plasmids and transgenic lines for studying localization of LT-B using GFP as a reporter. Gene cassettes A – E were used in transient assays using *Arabidopsis* leaf and root protoplasts, and in stable transformation of *Arabidopsis* seedlings

and maize callus and endosperm tissues. Constitutive expression in *Arabidopsis* protoplasts and seedlings, and in maize callus was driven by the double CaMV 35S promoter (P35S promoter). The maize 27 kD γ -zein promoter (P γ zein promoter) was used to drive expression of gene cassettes A – E in maize endosperm. The number of independent transgenic events for each line is presented in parenthesis. TEV, tobacco etch virus translational enhancer leader sequence; EGFP, enhanced green fluorescent protein; Tvsp, the soybean vegetative storage protein terminator; BSP, the LT-B bacterial signal peptide; LT-B, the B subunit of *E. coli* heat labile enterotoxin; ZSP, 27 kD γ -zein signal peptide; AG linker, alanine-glycine linker; na, not available.

Figure 2. Gene expression analyses of LT-B, GFP and LT-B::GFP fusions in transgenic maize kernels. (A), Bright field and fluorescence imaging of a representative self-pollinated ear of transgenic line P310 expressing GFP in the endosperm. A GFP-expressing kernel is marked by a white arrow. (B), LT-B levels as percent of total aqueous protein (% LT-B/TAEP) in endosperm of P310 and P311 kernels. (C), Western blot of TAEP extracts from transgenic callus (P308c) and endosperms (P309, P310-28, P310-32, P311 and P315) using anti-GFP antibody. (D), Western blot of TAEP extracts from transgenic callus (P308c) and endosperms (P309, P310-32, and P315) using anti-LT-B antibody. (E), Western blot of immuno-precipitated samples using anti-LT-B antibody, probed with anti-GFP antibody. Arrowheads in (C), GFP. Dots in (C), possible cleavage peptides cross react to GFP antibody. Asterisks in (C), (D) and (E), LT-B::GFP fusion. Open diamonds in (D), LT-B monomer. Open circle in (D), LT-B multimer. Arrow in (E), commercial EGFP.

Figure 3. Association of LT-B and GFP with starch and fiber fractions of transgenic maize kernels. (A), Arbitrary fluorescence per microgram total aqueous extractable protein (TAEP). (B), LT-B content as percent of TAEP. (C), Anti-GFP Western blot of starch soluble (TAEP) and insoluble (pellet) phases. (D), Anti-GFP Western blot of fiber soluble (TAEP) and insoluble (pellet) phases. Arrowheads, GFP. Asterisks, LT-B::GFP fusion. Dots, possible cleavage peptides cross react to GFP antibody.

Figure 4. Western blot analysis of total proteins from starch samples treated with Thermolysin. Samples were separated on a 12% SDS-PAGE, transferred to a 0.45 μ m nitrocellulose membrane, and probed with goat anti-GFP antibodies (A), rabbit anti-waxy protein antibodies (B), or rabbit anti-27 kD γ -zein protein antibodies (C), respectively. In panel A, arrowheads, GFP; asterisks, LT-B::GFP fusion; dots, possible cleavage peptides cross react to GFP antibody; arrows, Thermolysin-sensitive GFP band from P315 fiber fraction. Block arrow in (B), waxy protein. Open block arrows in (C), zein proteins.

Figure 5. Subcellular localization of GFP and LT-B::GFP fusions in transiently and stably transformed Arabidopsis and maize. Constructs A – E are described in Figure 1. Transiently transformed Arabidopsis leaf (a, f, k, p, u) and root (b, g, l, q, v) protoplasts using the constitutive P35S promoter constructs were imaged 24 – 48 hours after transformation. Stably transformed Arabidopsis seedlings (c, h, m, r) and maize callus (d, i, n, s, w) also used the P35S promoter constructs. Fresh immature endosperm (12-26 days after pollination) from transgenic maize seed carrying the P γ -zein promoter constructs were excised and imaged (e, j, o, t). Images are presented as merged green and red channels for all samples. Green signal in all images corresponds to GFP. Red signal in leaf protoplasts (a, f, k, p, u) and seedlings (c, h, m, r) is the autofluorescence of chlorophyll in chloroplasts. Red signal in root protoplasts (b, g, l, q, v) corresponds to the expression of a VirD2::RFP construct, a nuclear marker. Red signal in maize callus (d, i, n, s, w) and endosperm samples (e, j, o, t) is propidium iodide used as a counter stain that labels nucleic acids. Organelle labeling: chloroplasts (cl), cytosol (cy), nucleus (nu), endoplasmic reticulum (er), vacuole (va), starch (st). Bars = 10 μ m.

Figure 6. Co-localization experiments in Arabidopsis root protoplasts. Protoplasts were co-transformed using the constructs presented in Fig. 1 and an ER marker protein fused to RFP, ER cherry (Nelson, 2007). (A), pLM01 (GFP control). (B), pLM02 (BSP-GFP). (C), pLM03, (BSP-LT-B::GFP). (D), pLM08 (ZSP-LT-B::GFP). (E), pLM09 (LT-B::GFP). (F), pLM01 alone. (G), ER cherry alone. Green channel corresponds to GFP signal. Red

channel corresponds to ER-cherry signal. Merged images are also presented. Organelle labeling: cytosol (cy), nucleus (nu), endoplasmic reticulum (er). Bars = 10 μm .

P35S			Py-zein
Arabidopsis transient	Arabidopsis stable	Maize callus	Maize seed
pLM01	AtLM25 (5)	P307 (11)	P309 (10)
pLM02	AtLM26 (10)	P314 (12)	P315 (17)
pLM03	AtLM27 (3)	P308 (13)	P310 (16)
pLM08	AtLM28 (6)	P316 (12)	P311 (21)
pLM09	AtLM29 (6)	P317 (13)	P318 (na)

Figure 1.

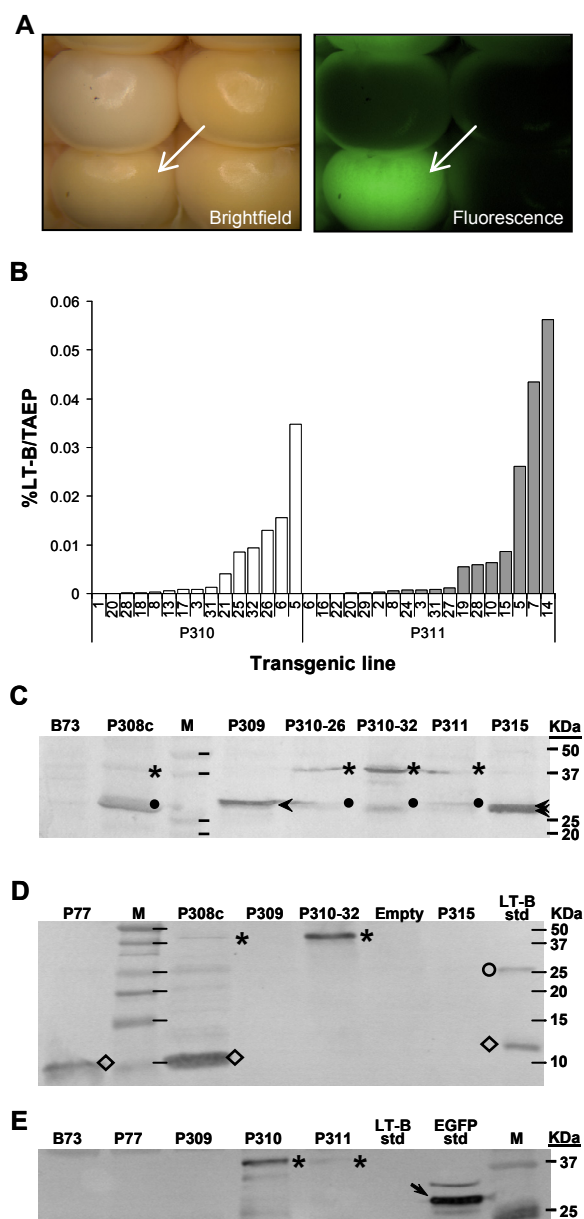


Figure 2.

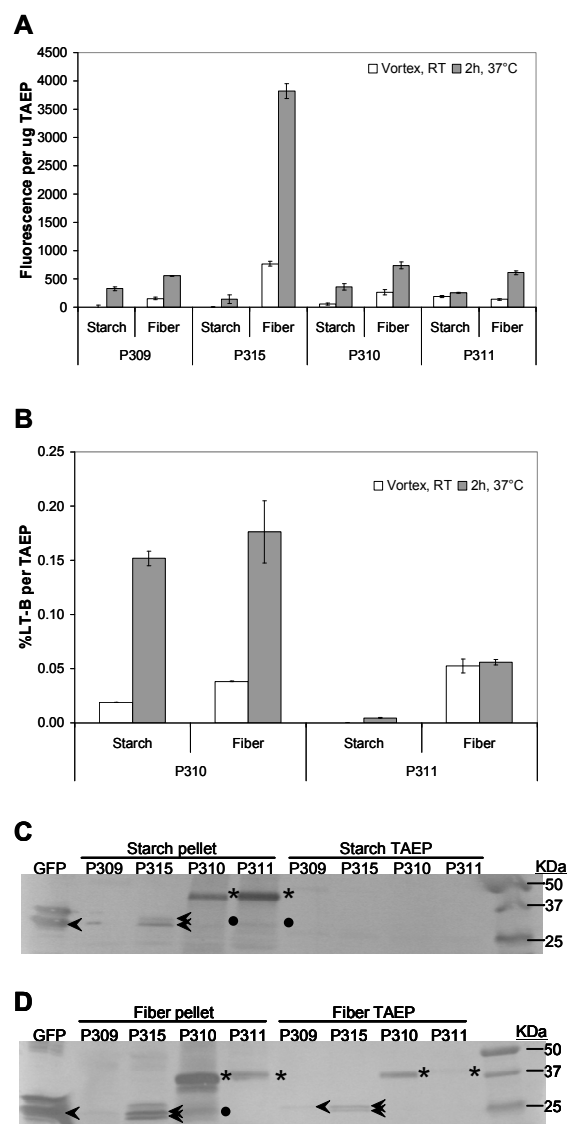


Figure 3.

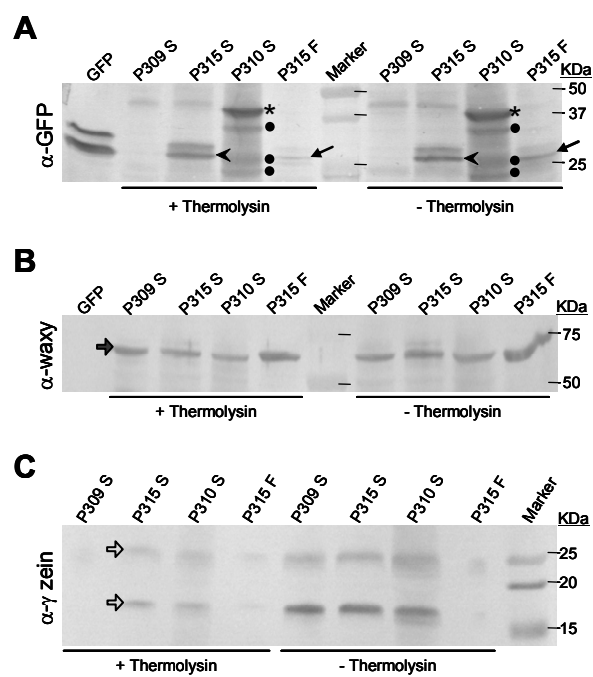


Figure 4.

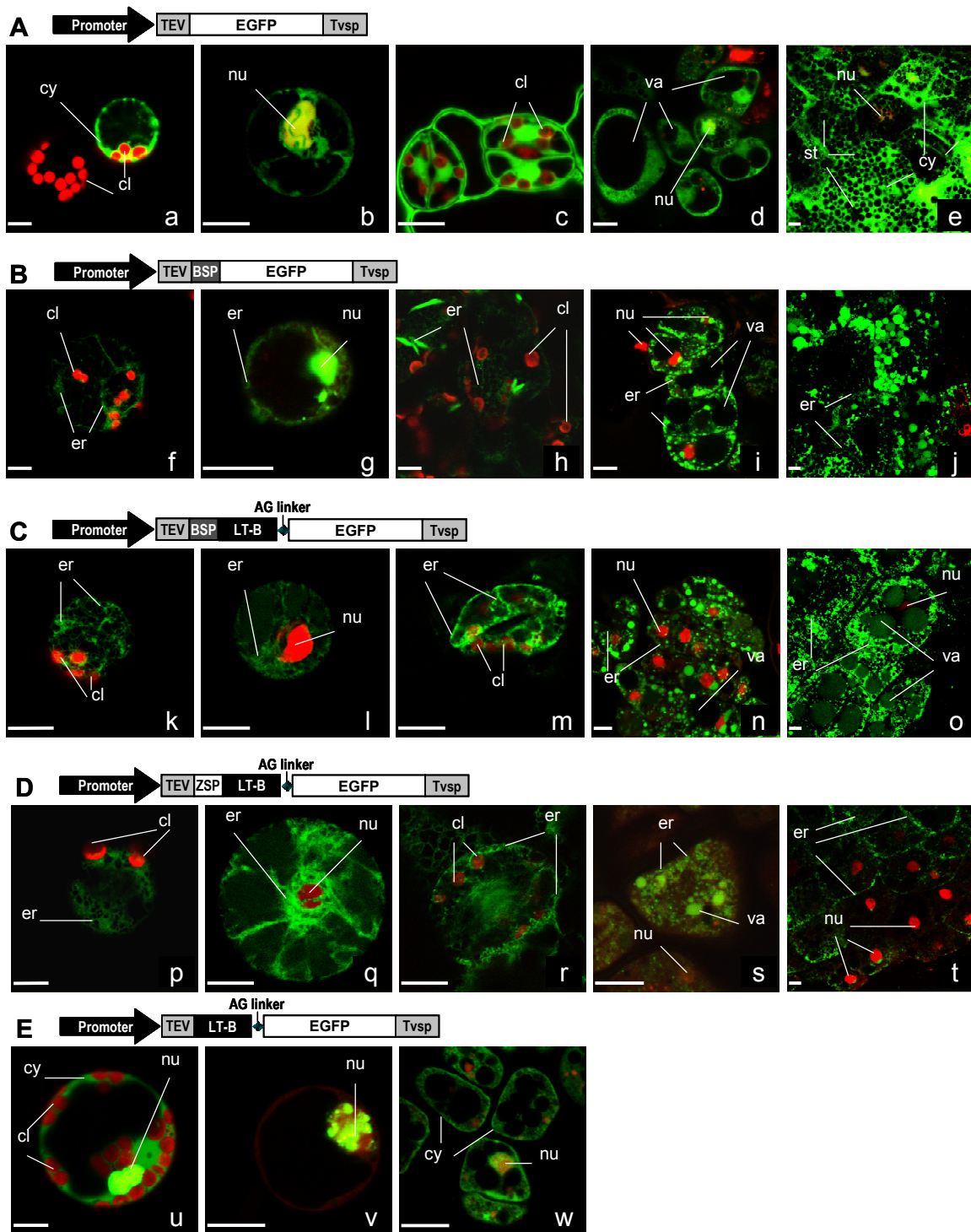


Figure 5.

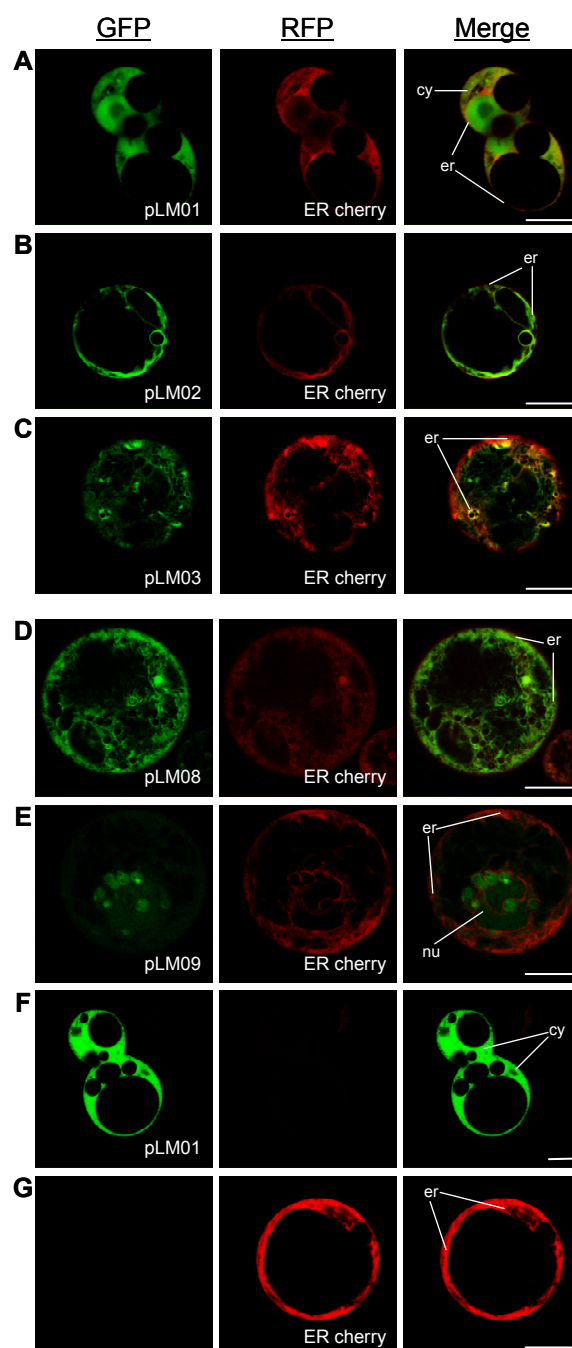


Figure 6.

CHAPTER 4: WET MILLING TRANSGENIC MAIZE SEED FOR FRACTION ENRICHMENT OF RECOMBINANT SUBUNIT VACCINE

Lorena Moeller, Raye Taylor-Vokes, Steven Fox, Qinglei Gan, Lawrence Johnson and
Kan Wang

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Abstract

Production of recombinant proteins in plants continues to be of great interest for prospective large-scale production of industrial enzymes, nutrition products and vaccines. This work describes fractionating by wet milling transgenic maize expressing the B subunit of the heat-labile enterotoxin of *Escherichia coli* (LT-B), a potent immunogen and candidate for oral vaccine and vaccine components. The LT-B gene was directed to express in seed endosperm tissue by an endosperm specific promoter. Two steeping treatments, traditional steeping (TS, 0.2% SO₂ + 0.5% lactic acid) and water steeping (WS, water only), were evaluated to determine effects on recovery of functional LT-B in wet-milled fractions. The overall recovery of the LT-B protein from WS treatment was > 1.5-fold greater than that from TS treatment. In both steeping types, LT-B was distributed similarly among the fractions, resulting in enrichment of functional LT-B in fine fiber, coarse fiber and pericarp fractions by concentration factors of 1.5 to 8 relative to the whole kernels, depending on the steep type and LT-B recovery units. Functional LT-B can be concentrated in wet-milled fractions of transgenic maize, preferably using water only for steeping. Combined with endosperm-specific expression of the recombinant protein, wet milling enables enrichment of high-value proteins in low-value fractions, such as the fine fiber, and the utilization of remaining fractions for traditional uses such as biorefining starch to biofuels and industrial chemicals.

Introduction

Transgenic plants offer a highly attractive platform for the production of therapeutic proteins, enzymes and value-added compounds, and the topic has been extensively reviewed [1-4]. The use of maize as a biofactory for producing recombinant proteins has been studied for nearly a decade now [5-7] and continues to be of great interest because of the numerous advantages it presents compared to bacterial, yeast or mammalian counterparts and high potential for commercialization. The infrastructure for producing, harvesting, transporting, storing and downstream processing of maize is well established. As one of the most cultivated crops of the world [8], maize production is also less costly than alternative bioreactors. As a non-bacterial and non-mammalian system, the use of maize for producing recombinant proteins greatly reduces the possibility of human pathogen contamination of products. As a higher eukaryote, maize is also equipped with the metabolic machinery to carry out post-translational modifications that influence the activity and nature of the recombinant protein of interest [9].

The maize kernel is composed of discrete tissues that include the embryo or germ, pericarp, bran and endosperm [10]. Successful production of recombinant proteins in maize has been reported for many kinds of proteins, from industrial enzymes and proteins [11-13] to alternative plastics [14] and edible vaccines [15-17]. Researchers have utilized a wide selection of promoters to drive expression of genes of interest in particular tissues, organs or developmental stages. The two most widely used tissues for recombinant protein expression are the germ [12, 13, 18] and the endosperm [19, 20]. The smaller amount of germ per kernel (11%) on mass basis compared to endosperm (82.9%) could be a potential advantage for achieving greater recombinant protein concentration. The endosperm, however, is a natural storage organ for storage proteins (primarily water-insoluble zeins) and starch represents up to 83% of the total kernel mass, which also makes it an attractive target for foreign protein accumulation.

Maize fractionation is traditionally accomplished by using dry- or wet-milling procedures. Dry milling with degerming allows recovery of bran-rich, germ-rich and endosperm-rich (of different grit sizes) fractions by physical and mechanical means. On the other hand, wet milling can result in recovery of six solid fractions that include the germ,

gluten meal, pericarp, coarse fiber, fine fiber, starch, and steep water [10, 21]. While dry-milling procedures are suitable for separating the germ and recovering endosperm with low oil and fiber contents, wet milling offers the advantage of recovering other fractions, such as highly purified starch and maize gluten meal, that can be used for other applications. Traditional wet milling involves steeping with 0.2% SO₂, which is key in breaking the cross-linking disulfide bonds in the endosperm protein matrix, producing acidic conditions that favor *Lactobacillus* growth (lactic acid softens the grain for grinding) and eliminating putrefying bacterial growth [22, 23]. The presence of SO₂ and lactic acid in the steep water increases release of proteinaceous material from the maize kernels [22] and enhances starch recovery [24]. Although much effort has been focused on optimizing wet-milling conditions for maize fractionation, much less has been done to study the effects of wet milling on recombinant protein recovery from transgenic maize. Fractionation studies for recovery of recombinant proteins have been carried out for germ-targeted [12, 25-27] and endosperm-targeted proteins [20, 25, 28] mainly using dry-milling or other non-wet-milling procedures.

Transgenic maize seed expressing recombinant avidin in germ was fractionated using a custom-made dehuller/degermer. Combined sieving and aspirating allowed recovery of a germ-rich fraction, which was compared to whole grain for its stability at elevated temperatures that are used during grain processing operations [12]. Transgenic maize expressing β -glucuronidase (GUS) and avidin in germ was also fractionated either by hand or dry milling, with a majority of extractable recombinant proteins recovered in the germ fractions [26]. The fractions enriched in GUS and avidin were further processed for purification of the recombinant proteins. Preliminary fractionation of GUS produced in maize by using the traditional wet-milling process adversely affected recombinant GUS activity [27]. Therefore, dry fractionation and germ flotation processes were used for fractionating transgenic maize expressing GUS enzyme. Comparison of dry fractionation with germ flotation separation revealed that the flotation method yielded higher enzyme recovery with up to 80% of the recombinant GUS activity accounted for [27]. Dry milling and hand fractionation of transgenic maize expressing the green fluorescent protein under control of an embryo- or endosperm-specific promoter were used to study the efficiency of the fractionation procedures [25]. Standard dry-milling procedures were also used to study

the benefit of fractionation in purification of a recombinant dog lipase targeted to be expressed in the endosperm of transgenic maize [20]. In this study it was reported that recombinant lipase can be extracted from both endosperm and germ fractions, but utilization of endosperm fraction can eliminate the need for germ defatting and its effects on recombinant protein purification.

Transgenic maize has been developed to express the B subunit of the heat labile enterotoxin from *Escherichia coli* (LT-B). Maize-derived LT-B has been shown to have potential as a vaccine or vaccine component when administered orally to mice due to its potent antigenic nature [17, 29]. Fractionation studies of LT-B maize have been conducted to determine whether orally delivered antigens can withstand the common commercial fractionation processes [15, 30]. Constitutively expressed LT-B maize was used for dry-milling fractionation, and the LT-B-rich germ fractions were further processed for utilization in feeding studies showing that LT-B is able to survive the dry-milling process while maintaining its antigenicity [15, 30].

The objective of the current study is to establish a fractionation procedure that will allow one to obtain fractions enriched in functional LT-B protein and remaining fractions suitable for other industrial applications such as biofuel (ethanol) production. We chose to evaluate the wet-milling process because it can recover six different fractions instead of three fractions compared to the dry milling-process, which we hypothesized would enable recovering fractions with greater LT-B concentrations that would make downstream purification easier and less costly. Due to the unknown effects of elevated steeping temperatures and SO₂/lactic acid used in traditional steeping on maize derived LT-B, we performed wet-milling fractionation using traditional steeping (TS; 0.2% SO₂ + 0.5% lactic acid) and water steeping (WS; water without SO₂ or lactic acid). Mass balance data for both steep types shows that there are significant differences in the recovery of coarse fiber, fine fiber, gluten meal and steep water fractions. Water steeping yielded a better recovery of functional LT-B overall than did traditional steeping. Both steep treatments result in enrichment of the fine fiber fraction with LT-B, a highly desirable output for currently a low value fraction. Because the starch fractions obtained from these steep treatments have very low levels of LT-B, it may also be ideal for traditional uses of starch, such as biorefinery or

fermentation for ethanol production. Altogether these results show that wet milling of LT-B maize is an adequate fractionation method for recovery of functional LT-B and has potential for co-production of starch and other fractions for industrial purposes.

Materials and Methods

Transgenic maize

Transgenic maize expressing LT-B was generated at the Center for Plant Transformation at Iowa State University (ISU) and designated as transgenic line P77 (LT-B maize; [19]). This line expresses LT-B in the endosperm. The maize used for this study was grown in Colorado under a regulated field release (U.S. Department of Agriculture, Animal and Plant Health Inspection Service field release permit # 04-131-01r) during the summer of 2004.

Fractionation of LT-B maize

Wet milling of transgenic maize was performed at the Center for Crops Utilization Research Unit at ISU using a modified procedure of that described for 100 g wet milling of maize [23] and detailed further in [31]. Steeping was carried out using 0.2% SO₂ and 0.5% lactic acid for traditional steeping (TS) and water only for water steeping (WS). Two 100-g samples were used for each steep condition, generating two replicates for each fraction recovered. All solid fractions recovered were dried, ground using a coffee grinder and sieved using a stainless-steel 40- μ m-mesh screen to achieve homogeneity before analysis. Each fraction recovered was analyzed for LT-B content in quadruplicate.

Protein extraction

For assaying LT-B, sieved solid samples were incubated with 10 μ L buffer per mg of dry matter containing 25 mM sodium phosphate (pH 6.6), 100 mM NaCl, 0.1% Triton X-100 (v/v), 1 mM ethylene-diamine-tetra-acetic acid (EDTA), 10 μ g/mL of leupeptin, 0.1 mM serine protease inhibitor Perfabloc SC, for 2 h at 37°C. Total aqueous extractable protein (TAEP) was determined by using the Bradford assay [32]. Protein extraction for Western blots was carried out at 50°C using the same buffer.

LT-B detection by G_{M1} capture ELISA

Quantification of LT-B in the samples was carried out using a modification of the monosialoganglioside (G_{M1}) dependent enzyme-linked immunosorbent assay (ELISA) described previously [33]. The described protocol was modified as follows. Monosialoganglioside G_{M1} from bovine brain (G7641, Sigma, St Louis, MO, USA) was used at a 10 $\mu\text{g/mL}$ concentration, 50 μL per well. Streptavidin-horseradish peroxidase conjugate (554066, BD Biosciences, San Jose, CA, USA) was used at a dilution of 1:1000 in 1% dry milk (DM) (w/v) in phosphate buffered saline [PBS; 0.01 M Na_2HPO_4 , 0.003 M KH_2PO_4 , 0.1 M NaCl, (pH 7.2)]. Horseradish peroxidase substrate [ABTS; 0.5 mM 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 0.1 M citric acid, pH 4.35] was activated prior to use, by adding 5.5 μL 30% H_2O_2 to 5.5 mL ABTS solution. Activated horseradish peroxidase substrate was added to the plate and incubated in the dark at room temperature for 30 min. Absorbance was measured spectrophotometrically at 405 nm, at the end of the reaction. Sample wells were blanked against non-transgenic maize protein extracts and all measurements were performed in duplicate (technical replicates). Raw ELISA data was converted using an ELISA standard curve constructed using purified bacterial LT-B (kindly provided by Dr. John Clements, Tulane University, New Orleans, LA, USA). LT-B content was expressed as μg LT-B/g solids or % LT-B/ TAEP.

LT-B detection by Western blotting

Equal amounts of TAEP were diluted in 2x Laemmli SDS-sample buffer [34] and boiled for 5 min. Samples were separated on 18% polyacrylamide SDS-PAGE gels as described [34]. The separated proteins were transferred to a 0.45 μm nitrocellulose membrane using the BioRad Semidry Transblot apparatus according to the manufacturer's instructions. Unless otherwise specified, all incubations were carried out at 25°C for 1 h. Membranes were blocked with 5% DM in PBST (PBS - 0.05% Tween-20 [v/v]). Rabbit anti-LT-B (RECO-55G, Immunology Consultants Laboratory, Inc., Newberg, OR, USA) was used as primary antibody at a 1:1000 dilution in 1% DM in PBST. A horseradish peroxidase conjugated goat anti-rabbit (A0545, Sigma, St Louis, MO, USA) was used as secondary antibody at a 1:2000 dilution in PBST. Colored bands were revealed by incubation with

horseradish peroxidase substrate, 3,3',5,5'-Tetramethylbenzidine (T0565, Sigma, St. Louis, MO, USA) and dried membranes used for imaging.

Data analysis

Mass balance data were analyzed in Excel by using t-tests for mean comparisons. LT-B data for wet milling were analyzed using Proc Mixed code in SAS ($\alpha = 5\%$).

Results and Discussion

The expression of the LT-B in maize has been accomplished by using different types of promoters to regulate protein production in different maize tissues. The endosperm-specific promoter leads to the LT-B production specifically in the seed endosperm tissue [19] while the embryo-enhanced promoter or constitutive promoter gives embryo (or germ) enriched LT-B expression [16]. Maize-derived LT-B from both expression strategies has been shown to be effective in mice and humans protecting against traveler's diarrhea caused by enterotoxigenic strains of *E. coli* [15, 17, 30]. The LT-B in these studies, however, was either processed as a whole maize product [17] or as a germ-enriched product [15, 30]. No wet-milling process for recovery of edible vaccines or vaccine components from maize has been reported. Most grain fractionation processes for recovery of recombinant proteins produced in maize have been based mainly on hand separation or dry-milling procedures [12, 15, 20, 26, 27, 30]. On the other hand, an early preliminary study on fractionation of transgenic maize producing β -glucuronidase (GUS) by wet milling showed detrimental effects on recombinant GUS activity [27]. In order to study the effects of the steeping composition on LT-B recovery from wet-milled fractions, we carried out the milling process in duplicate using traditional (SO_2 + lactic acid, TS) and water steeping (WS).

Comparison of mass recovery from wet milling of LT-B maize using traditional and water steeping

Table 1 presents the mass balance data for TS and WS wet-milled fractions of LT-B maize. The values are the means of the two replicates for each treatment. Yields of starch, germ and pericarp fractions were not significantly different between the two steep types. On the other hand, the yields of gluten meal, coarse and fine fiber, and steep water fractions were

significantly different between traditional and water steeping. Gluten meal and steep water recovery were higher using traditional steeping, while recovery of coarse and fine fiber were higher using the water steep. Addition of SO₂ + lactic acid to the steeping process prevents colonization of undesirable microorganisms but allows growth of *Lactobacillus* for lactic acid synthesis, which helps in kernel hydration and swelling, promotes kernel enzyme activation, and aids in deconstructing the protein matrix by reduction of disulfide bonds and increased protein solubility. Traditional steeping also reduces protein interactions with starch, which results fractions in higher purity, a desired characteristic for downstream processing [10, 21].

Starch and germ fractions are usually composed of 0.3 and 12% protein on dry basis (db), respectively [10, 21]. The SO₂ + lactic acid affect mostly the endosperm of the maize kernel, from which the other fractions are derived. In the commercial wet-milling process, gluten meal and steep water fractions typically contain 60 and 46% protein db, respectively. Coarse and fine fiber collectively are composed of 12% protein db, and higher recovery of these fractions in water steeping may be due to less accessibility and protein deconstruction of the endosperm material in the absence of SO₂ + lactic acid [10, 21]. Our data indicate that the yield recoveries from both steeping treatments are in line with that reported for the wet-milling processes [10, 21].

LT-B distribution in wet-milled fractions

We evaluated the recovery of functional LT-B in each fraction collected from both steeping treatments. LT-B is synthesized as a monomer protein (11.6 kD) that assembles into a homopentamer given the proper environment in the native bacterial periplasm [35], or the heterologous plant compartment where it accumulates [33, 36]. The pentameric form is recognized specifically by the host's intestine receptors, the monosialoganglioside G_{M1} receptor, to internalize the toxin and mediate the immune response [37]. Therefore, as a potential subunit vaccine or vaccine component, LT-B's functionality depends on its successful assembly into a functional pentamer. While LT-B protein can be detected in different forms using a sandwich ELISA with LT-B specific antibodies [38], in this study, we focused on the recovery of the functional pentameric LT-B protein.

Table 2 presents the distribution of functional LT-B in each of the recovered wet-milling fractions for each of the two steeping methods. The values reported for coarse fiber, fine fiber, germ, gluten meal, pericarp and starch correspond to the least squares means for each fraction and steep method combined, while steep liquids and whole kernel data represent the averages of four readings.

The field-grown LT-B maize seed has LT-B level measured as 28.44 μg LT-B per g of seed when analyzed as whole kernel ground meal sieved in the same manner as the solid fractions recovered from wet milling. The same batch of maize used for fractionation was used as a sample for whole kernel processing. As can be seen in Table 2, the traditional steeping and water steeping gave similar distributions of LT-B in the different fractions. The functional LT-B is concentrated in the fine fiber fraction obtained from both treatments. Detectable levels of functional LT-B were observed in all fractions, with the germ and starch being the lowest LT-B containing fractions. No significant differences were observed between steep treatments for LT-B contents in wet-milled fractions for any of the fractions, except for fine fiber levels expressed in per mass basis.

Table 2 also shows the functional LT-B recovery in each of the wet-milled fractions. In general, the trend observed for LT-B content is conserved, with the fine fiber, coarse fiber, gluten meal and pericarp having the highest recoveries of LT-B per g of maize fractionated. A total of 12.71 μg LT-B was recovered per g of maize fractionated using traditional steeping, representing 44.64% of whole kernel LT-B (28.44 $\mu\text{g/g}$). On the other hand, 20.27 μg LT-B were recovered per g of maize fractionated using water steeping, accounting for 71.17% of the total LT-B in whole kernel.

This result suggests that TS may have negative effects on recombinant protein recovery, as was observed for GUS activity [27] and LT-B [39]. Addition of SO_2 and lactic acid enable breakage of cross-linking disulfide bonds in the endosperm protein matrix [22, 23], and possibly of the recombinant protein as well. In the case of LT-B and of most proteins, correct folding and functionality depends highly on disulfide bond bridges within the protein itself. It is likely that SO_2 and lactic acid directly affect LT-B protein stability, folding and assembly into functional pentamers. The high recovery of functional LT-B observed from

wet milling using water steeping suggests this method is more adequate than TS for high recovery of functional LT-B in wet-milled fractions.

Table 2 shows that in terms of LT-B yield, however, both steeping treatments result in 20-30% recovery in fine fiber in terms of whole kernel LT-B (28.44 μg LT-B/g) and 40-45% recovery in terms of total recovered LT-B (12.70 μg LT-B/g for TS, and 20.24 μg LT-B/g for WS).

Table 3 shows that LT-B can be concentrated in the fine fiber fraction recovered by wet milling by a factor of 4 to 8, depending on the steeping treatment. Fine fiber represents 2.48 and 7.06% of kernel mass for TS and WS (Table 1), respectively. LT-B is therefore highly concentrated in a relatively small amount of mass, which is favorable for further processing of the enriched fraction and enhanced vaccine potency, as has been shown to be the case of dry milling LT-B-germ-enriched fractions in previous studies [15].

Coarse fiber, gluten meal, pericarp and steep liquor also contain LT-B and represent a combined 34.62 and 33.13% kernel mass for TS and WS, respectively. There are no significant differences in LT-B recovery for these fractions using TS or WS steeping treatments. Maize gluten meal, fiber and steep liquid are low-value fractions usually sold as maize gluten feed for the livestock industry [10, 21].

Even smaller amounts of LT-B were detected in germ and starch fractions recovered from both TS and WS in wet milling. LT-B levels in the germ and starch are lowest compared to the other wet-milled fractions. The low accumulation of LT-B in germ is due to the specific expression of the protein in the endosperm tissue of seed [19].

Utilization of germ and starch fractions recovered from either wet or dry milling is well known and include oil extraction from germ and highly purified starch [10] for food, paper coatings, adhesive etc. [10, 21]. Downstream processing of germ for oil extraction generally requires prepressing prior to solvent extracting with hexane. The fate of LT-B in wet milling remains to be tested to determine if the germ fraction product of wet milling endosperm-targeted LT-B maize is utilizable for traditional oil recovery. On the other hand, the lack of enrichment of LT-B in the starch fraction would allow the utilization of starch in downstream processes such as fermentation to produce biofuel and industrial chemicals. This result is

highly desirable considering that starch represents 59.98 and 56.78% of kernel mass in TS and WS wet milling, respectively (Table 1).

Our data indicate that even though the distribution of LT-B in recovered fractions was similar with TS and WS, the total recovery of functional LT-B was higher with WS. In addition, LT-B was enriched in the fine and coarse fiber fractions. Both fractions were recovered at significantly higher levels from WS (Table 1), probably due to adhering starch. Therefore, water steeping is a preferred approach for extracting and concentrating endosperm-expressed LT-B from maize seed.

Detection of monomeric LT-B in wet-milled fractions

Fig. 1 presents a Western blot of TAEP from fractions obtained from wet milling of TS and WS treated transgenic LT-B maize. Because G_{M1} -specific ELISA can only capture functional LT-B when it is in pentameric form, the Western blot is used here to detect all forms of LT-B proteins, typically the monomeric LT-B in a denaturing SDS gel. Fig. 1 shows that in general, the levels of functional LT-B observed using G_{M1} capture ELISA in all fractions corresponds with the detection of monomeric LT-B by Western blotting. Fig. 1 also shows that at equal total extractable protein levels across fractions, WS fractions have higher levels of LT-B. As mentioned previously, it is possible that LT-B exists in a variety of forms from monomers to pentamers. It has been reported that the level of functional pentameric LT-B detected by G_{M1} ELISA is within 40% of the level detected using a sandwich ELISA that detects total LT-B protein [16]. In the present work, we focused on assessing pentameric LT-B, as this is the functional form of the protein for G_{M1} receptor – mediated internalization of the protein for immune response elicitation.

Conclusions

Fractionation of transgenic LT-B maize by traditional wet milling (steeping in 0.2% SO_2 + 0.5% lactic acid) and water steeping results in significantly different mass recoveries for coarse fiber, fine fiber, gluten meal and steep water fractions (fractions associated with high protein content). No effect of steeping treatment was observed for recovery of germ, pericarp and starch fractions.

Analysis of functional LT-B content in wet-milled fractions showed that functional LT-B was detected in all fractions regardless of steeping conditions; however, greatest enrichment of functional LT-B was in the fine fiber fraction for both steeping treatments. Fine fiber fractions from wet-milled transgenic LT-B maize were enriched up to a factor of 8.2 when compared to the level in whole kernels and present great potential for further utilization as a vaccine or vaccine component. With a relatively low protein content of 12%, LT-B enrichment in the fine fiber fraction represents potentially reduced maize protein impurities for utilization as oral vaccine or downstream processing or purification of LT-B. One of the remarkable properties of maize-derived LT-B is its potent immunogenicity when delivered orally [40]. Therefore, the enriched fine fiber fraction, a low-value fraction, shows enhanced added value due to its potential use as a direct delivery system for LT-B. Previous human trials for plant-derived LT-B have used dosage corresponding to 0.75 – 1 mg LT-B per dose delivered as an enriched germ product of maize [30] or as potato tuber [41]. Even with the moderate LT-B expression level in the line used for this work, this dosage can be easily achieved from the fraction enrichment process employed. Furthermore, expression levels can further be increased through breeding and selection to achieve even higher dosage. Another major benefit of using LT-B enriched fractions from wet-milling is the potential use of spent fractions for other applications, allowing some traditional uses of germ and starch fractions in downstream processing.

In addition, it is conceivable to design future strategies for recovery of different recombinant enzymes from different fractions of the wet-milling process. The information presented in our work can help shape strategies for designing specialty crops of tomorrow. The recovery of functional LT-B in fractions by using conventional wet-milling procedures enhances the potential use of maize as a bioreactor for vaccine or vaccine components. The enrichment of LT-B in the fine fiber fraction reduces significantly the quantity of material necessary to achieve a particular dose of antigen. Wet milling, therefore, has the potential to be a standard processing step for future applications of maize-derived LT-B vaccine and vaccine components. Based on LT-B and fraction recoveries observed for both types of steeping, we recommend that water be used instead of traditional SO₂ and lactic acid

(endogenously produced or added) steeping to prevent unnecessary loss of recombinant protein and help improve yield of LT-B enriched fractions.

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Table 1Mass balance data for recovered fractions derived from wet milling LT-B transgenic corn [†]

Fraction	Traditional Steep			Water Steep		
	Yield (% db) [§]	±	Standard error	Yield (% db)	±	Standard error
Coarse fiber	3.87 ^a	±	0.15	8.63 ^b	±	0.09
Fine fiber	2.48 ^a	±	0.09	7.06 ^b	±	0.29
Germ	2.76 ^a	±	0.09	3.12 ^a	±	0.31
Gluten meal	20.52 ^a	±	0.32	16.71 ^b	±	0.22
Pericarp	5.66 ^a	±	0.06	5.57 ^a	±	0.08
Starch	59.98 ^a	±	0.03	56.78 ^a	±	0.36
Steep liquids	4.57 ^a	±	0.07	2.22 ^b	±	0.02
Total solids	99.84	±	0.30	100.07	±	0.02

[†] Values followed by the same letter within a fraction are not significantly different (P<0.05)[§] Dry basis

Table 2

LT-B recoveries in wet-milled fractions of transgenic corn

Fraction	Traditional Steep				Water Steep			
	LT-B content	LT-B recovery			LT-B content	LT-B recovery		
		(μg LT-B / g solids)	(μg LT-B / g corn) ^a	% WK LT-B ^b		% total LT-B ^c	(μg LT-B / g solids)	(μg LT-B / g corn) ^a
Coarse fiber	39.85	1.54	5.42	12.15	41.40	3.57	12.56	17.65
Fine fiber *	234.53	5.82	20.45	45.81	115.14	8.13	28.58	40.16
Germ	9.56	0.26	0.93	2.08	15.01	0.47	1.65	2.31
Gluten meal	10.18	2.09	7.35	16.46	27.51	4.60	16.16	22.71
Pericarp	48.98	2.77	9.75	21.84	59.35	3.31	11.62	16.33
Starch	0.05	0.03	0.03	0.08	0.06	0.04	0.04	0.05
Steep liquids	4.40	0.20	0.71	1.58	7.13	0.16	0.56	0.78
Total		12.71	44.64	100.00		20.27	71.17	100.00

^a μg LT-B / g corn is calculated as following: (fraction LT-B content x fraction mass yield)/100^b % of LT-B per mass is calculated as following: (fraction LT-B content x fraction mass yield / 28.44)^c % of LT-B per mass is calculated as following: (fraction LT-B content x fraction mass yield / total recovered LT-B)* LT-B content significantly different ($\alpha=0.05$) for Traditional and Water steeping

Table 3

Concentration factors of LT-B achieved through the wet-milling process

Fraction	$\mu\text{g LT-B per g ground solids}$		% LT-B / TAEP	
	Traditional steep	Water steep	Traditional steep	Water steep
Coarse fiber	1.4	1.5	1.2	0.7
Fine fiber	8.2	4.0	5.8	5.2
Germ	0.3	0.5	0.2	0.2
Gluten meal	0.4	1.0	0.6	0.8
Pericarp	1.7	2.1	1.7	1.2
Starch	0.0	0.0	0.0	0.0
Steep liquids	0.2	0.3	0.2	0.3
Whole kernel	1	1	1	1

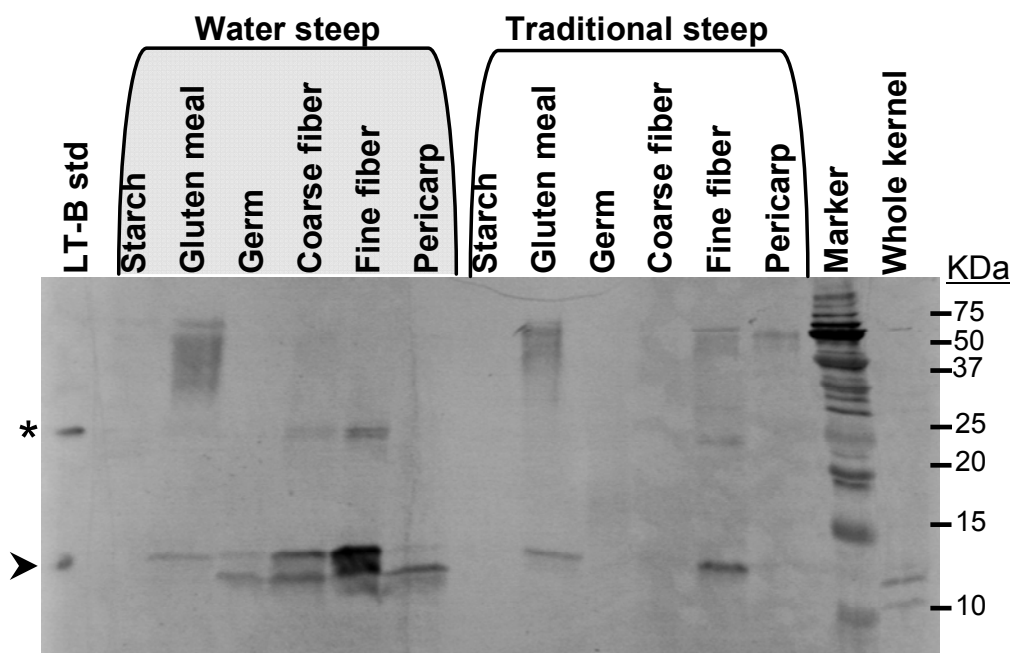


Figure 1. LT-B Western blot from wet-milled fractions. Equal amounts of TAEP were loaded for all samples. LT-B std, bacterial LT-B used as control. Arrowheads, LT-B monomer (~11.6 kD). Asterisks, LT-B multimers.

CHAPTER 5: ESTABLISHMENT AND CHARACTERIZATION OF A MAIZE HI II ENDOSPERM CULTURE

Lorena Moeller, Qinglei Gan and Kan Wang

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Abstract

An *in vitro* continuous endosperm callus (ENC) culture derived from 11-day-after-pollination developing endosperm of transformation-amenable maize Hi II genotype is reported. The ENC established in this work has cells that differentiate into aleurone-like and starchy endosperm-like cell types and can be maintained for a course of four years. ENC cells transcribe and produce zein proteins at a level similar to developing endosperm tissue. Starchy endosperm cells of ENC have active starch biosynthetic enzymes and synthesize starch. The dual cell physiology of this culture limits the utility for promoter analysis and transient assays of gene expression in the current culture conditions. However, because the system can be readily initiated and easily maintained for a long period of time, it provides an alternative tool for analysis of transgene expression in ENC derived from transgenic maize lines in Hi II background.

Introduction

As one of the three major crops of the world, maize or corn (*Zea mays*) is staple food, feed and resource for many human activities. Maize kernel is an important organ, composing > 80% of endosperm, a starch and protein reservoir designed to provide nutrition to the embryo at germination (Johnson 2000). The endosperm is the part of the kernel most used by humans for food, feed, and fuel applications and as such, is not only an attractive tissue for quality improvement and recombinant protein production, but is also a vital tissue for basic research on seed development. The maize endosperm has been studied for several decades now, and more recently, has been the focus of biotechnological approaches to diversify its use by humans. Efforts have been made to alter the endosperm's protein composition (Crow and Kermicle 2002) and starch quality and digestive properties (Ao, et al.

2007). Numerous reviews have summarized the potential use of maize as a bioreactor for production of recombinant proteins (Hood, et al. 1999; Kusnadi, et al. 1998; Ramessar, et al. 2008; Streatfield, et al. 2003) and biofuel potential (Dhugga 2007; Torney, et al. 2007).

Most molecular biology research on kernels has been carried out using mutants (Walbot 1991) or stable maize transformation in which seed-specific promoters drive the expression of gene of interest in different compartments of seeds (Shen and Petolino 2006). Either process is usually lengthy and laborious due to the extended period needed from planting to endosperm filling and the nature of the endosperm tissue itself which makes it less amenable to manipulations than other abundant tissues such as leaves and roots. Tissue culture of maize endosperms, therefore, has been the focus of extensive research for decades (Gruis, et al. 2007), with reports of successful *in vitro* growth of maize endosperms as early as 1949 (La Rue 1949). Since then, endosperm cultures have been analyzed for their culture requirements (Shannon and Liu 1977; Straus and La Rue 1954), spontaneous changes *in vitro* (Straus 1958), ultrastructure (Felker 1987), anthocyanin production (Racchi and Manzocchi 1988), sugar uptake in suspension cultures (Felker and Goodwin 1988), zein expression (Shimamoto, et al. 1983), and glycoprotein synthesis (Riedell and Miernyk 1988). *In vitro* endosperm suspension cultures were used for transient transformation analysis of zein gene regulation and chimeric gene expression (Quayle, et al. 1991; Ueda and Messing 1991). Recently, endosperm callus culture generated from a transgenic maize line was used for study of the aleurone cell specification in seed (Gruis, et al. 2006).

The genotypes used routinely for endosperm cultures up to date are limited. Very few lines have been analyzed thoroughly and most studies have been done using cultures derived from inbred line A636. The effect of inbred and hybrid genotypes on culture establishment (Shannon and Batey 1973) has been studied and it is known that not all genotypes of maize are amenable to endosperm callus culture. This is similar to the effect of genotype on tissue culture response derived from embryos (Armstrong, et al. 1991).

The purpose of this study is to establish a continuous *in vitro* culture of endosperm that possesses the characteristics of maize seed. Here we describe the generation and properties of a maize endosperm culture line derived from Hi II endosperm at 11 d after pollination. We chose the Hi II genotype for this work because it is a genotype amenable to maize

transformation. Seed-specific regulation study using transgenic maize kernels can be further advanced in a continuous *in vitro* endosperm culture to circumvent endosperm tissue limitation from single seed.

The endosperm culture reported here can be maintained *in vitro* continuously for over a three year period. Our biochemical and molecular biological characterization of this culture suggests that the Hi II maize endosperm culture described in this study displays similar properties to immature endosperm tissues with the ability to synthesize starch and zein proteins as well as cell differentiation into aleurone and starchy endosperm cells. This culture presents potential as a tool to enhance knowledge of endosperm development and recombinant protein production.

Materials and methods

Plant materials and tissue culture medium

Maize plants were grown in the greenhouse of the Agronomy Department at Iowa State University, under previously published conditions (Frame, et al. 2006). Plants were sib- or self-pollinated and ears were harvested at 11 days after pollination (DAP). Genotypes B73, B104 and Hi II were used to test for their ability to initiate callus formation from isolated endosperms.

Full strength solid Murashige and Skoog (Murashige and Skoog 1962) medium supplemented with 100 mg/L myo-inositol, 30 g/L sucrose, 2 g/L asparagine, 0.5 mg/L thiamine, 2.5 g/L gelrite, pH 5.8, was used after (Ueda and Messing 1991). Medium is identified as MSA hereafter.

Endosperm isolation, culture initiation & maintenance

Hi II ears harvested at 11 DAP were de-husked, surface sterilized using 50% commercial grade bleach and a drop of Tween-20 for 20 min, and rinsed three times with sterile water. A sterile scalpel blade was used to remove the caps of the kernels, one row at a time, and the endosperms were scooped using a sterile spatula onto fresh MSA medium, carefully avoiding the embryo or any maternal tissue. Plates were sealed with parafilm and incubated in the dark at 28°C. One to three months after the initial plating, callus was beginning to be visible

in some kernels. This callus was transferred onto new MSA plates and subcultured every 2-3 weeks.

Tissue preparation for microscopy

Tissue (11 DAP maize kernels and endosperm callus) was cut in cold fixative (0.1 M cacodylate buffer, 0.5% glutaraldehyde and 2% paraformaldehyde) to pieces 0.5 – 1 mm in size. Tissue blocks were incubated in fixative for 2 h at 4°C and rinsed three times in 0.1 M cacodylate buffer, 15 min each rinse, on a rotating shaker. Tissue blocks were dehydrated as follows: rinse with 50% ethanol for 15 min, followed by incubations with 70% and 95% ethanol for 2 h each, at room temperature. Tissue blocks were then incubated in 100% ethanol for three successive two-hour incubation periods. Tissue blocks were then incubated in a gradually increasing concentration of White London Resin (LR White), starting with 1:3, 1:1, 3:1 (vol:vol) LR White to ethanol for 8 – 12 h each time, and finally with 100% LR White overnight. Incubation in pure LR White was repeated twice for 8 – 12 h each time, after which the tissue blocks were cast in gelatin capsules for 48 – 72 h in a cold room (8 – 12°C), under UV light to polymerize. Semi-thin sections (1 µm) were cut using an ultramicrotome and glass knives and mounted onto Probe-On Plus slides.

Sections were stained using toluidine blue O and I₂/KI solutions for 10 min in the dark. Slides were washed three times with distilled water, and air dried. Cover slips were applied on the sections after addition of a drop of xylol and Permunt. Bright-field and phase contrast images were taken at Microscopy and Nanoimaging facility at Iowa State University. Images were processed using the AxioVision software.

Fresh endosperm slices or transgenic endosperm callus was mounted directly onto Probe-On Plus slides using VectaShield medium with propidium iodide. Images were taken using a laser scanning confocal microscope (Leica TCS/NT or Leica SP5 X (Leica Microsystems Inc., Exton, PA). Confocal micrographs were processed using ImageJ software and Adobe Photoshop.

RNA extraction and cDNA synthesis

Qiagen RNeasy kit was used to extract RNA from 11 DAP Hi II endosperms, Hi II endosperm callus, and Hi II embryo callus, following manufacturer's instructions. RNA

quality was checked using a 2% agarose gel. Invitrogen's SuperScript III First-Strand Synthesis Super Mix (cat# 18080-4000) was used for first strand cDNA synthesis, following supplier instructions.

Semi-quantitative PCR

Semi-quantitative polymerase chain reaction (PCR) was done using 0.5 μ L of cDNA as template for all PCR reactions. Primer sequences are listed in Table 1. Biolase DNA polymerase (Bioline BIO-21042) was used following supplier specifications. PCR products were analyzed in a 1% agarose gel stained with ethidium bromide and imaged on a UV transilluminator.

Protein extraction and electrophoresis

One-hundred mg of tissue (11 DAP endosperm, endosperm callus and embryo callus) were collected in 1.5 mL Eppendorf tubes. Samples were extracted using an aqueous extraction buffer (Aq; 25 mM sodium phosphate buffer pH 6.6, 100 mM NaCl, 1 mM ethylene-diamine-tetra-acetic acid, 0.1% Triton-X 100, 10 μ g/mL Leupeptin, 0.25 mM Pefabloc SC) or an ethanol based extraction buffer (Et; 75% Ethanol, 5% β -mercaptoethanol). Six-hundred μ L of extraction buffer were added to each tube, and samples were homogenized using a hand-held pestle. Samples were incubated at room temperature in a vortex shaker for at least 30 min and then were pelleted by centrifuging at 14,000 rpm for 10 min in a tabletop microcentrifuge. Supernatants were transferred to a fresh Eppendorf tube. For α -zein and waxy protein gels, samples were frozen in liquid nitrogen and lyophilized 48 h. Dried samples were resuspended in 200 μ L of 2x Laemmli SDS-sample buffer (Laemmli 1970). Thirty μ L of resuspended sample were diluted 1:1 with water and boiled for 5 min before loading 25 μ L per well in 12% polyacrylamide gels. For γ -zein, 100 μ L of each fresh sample was diluted 1:1 with 2x Laemmli SDS-loading buffer (Laemmli 1970), boiled for 5 min and 60 μ L were loaded onto two 12% acrylamide gels. One gel was stained with coomassie blue for total protein stain, and the other gel was used for western blot. Gels were electrophoresed at 110V for 20 min and 130V for 50 min and subsequently

washed in water. Protein gels were stained with coomassie blue for 1 h and de-stained in boiling water as needed.

Western blots

Gels for western blots were transferred to a nitrocellulose membrane using a semi-dry transfer apparatus (Biorad) at 10V for 30 min. Membranes were then washed with water, and then with phosphate buffered saline with Tween-20 (PBST; 0.01 M Na₂HP0₄, 0.003 M KH₂PO₄, 0.1 M NaCl, 0.05% Tween-20, pH 7.2) for 5 min. Membranes were blocked with 5% dry milk in phosphate buffered saline (PBS) for 1 h at room temperature, followed by one wash with PBST. Membranes were incubated with rabbit anti- α -zein (1:2000 in 1% dry milk in PBS) and rabbit anti- γ -zein (1:3000 in 1% dry milk in PBS) antibodies (P. Scott, USDA/ARS, Ames, IA) for 1 h at room temperature, followed by three washes with PBST, 5 min each. Secondary antibody, goat anti-rabbit IgG – alkaline phosphatase conjugate was added at 1:5000 dilution in 1% dry milk in PBS and incubated at room temperature for 1 h, followed by three washes with PBST, 5 min each. The membrane was then washed with alkaline phosphatase substrate buffer for 1 min before adding 20 mL of alkaline phosphatase substrate (Biorad). The membrane was incubated for 5-10 min until a signal was visible when the reaction was stopped by adding water. Membranes were air-dried overnight and photographed using a FujiPix digital camera.

Zymmograms

One hundred to 1000 mg of sample was homogenized using a handheld microfuge pestle with or without treated with liquid nitrogen. Samples were then extracted with Buffer 1 (25 mM sodium phosphate buffer, pH 6.6, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10 μ g/mL Leupeptin, 0.5 mM Pefabloc), or Buffer 2 (50 mM Tris-acetate, pH 7.5, 10 mM DTT). Fifteen micrograms of protein were mixed with native loading buffers (25% glycerol, 0.1% bromophenol blue; or 30% glycerol, 0.25% bromophenol blue) and loaded onto a native polyacrylamide gel containing 0.3% corn or potato starch. Electrophoresis was run using 1x Tris-Glycine native running buffer with or without 2 mM DTT. Gels were run for 2 h at 100V, incubated in renaturation buffer (100 mM Tris-Cl, pH 7.0, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM DTT) overnight at room temperature. Large (16 cm x 20 cm x 0.15 cm) native

gels for starch enzyme activity confirmation were performed as described (Dinges, et al. 2001). Finally, gels were stained with I₂/KI solution (1 g/10 g/L) until bands became visible. Gels were imaged using a FujiPix digital camera and a white light box.

DNA Constructs

Construct A (pAHC25; Christensen and Quail 1996) was a gift of Dr. P. Quail (University of California-Berkeley/USDA Plant Gene Expression Center). Construct B (Vp1P-Gus; Cao, et al. 2007) was a gift from Dr. P. Becraft (Iowa State University). Construct C (22 α z P-GUS) was generated by gateway recombination using entry vectors containing a maize 22 kD α -zein promoter (fl2; a gift from Dr. D. Jackson, Cold Spring Harbor Laboratories) the GUS gene, and the Nos terminator. Construct D (35S P-GFP; Mankin, et al. 2003) was a gift from Dr. W. Thompson (North Carolina State University). Construct E (27 γ z P-GFP; Shepherd, et al. 2008) was a gift from Dr. P. Scott (USDA/ARS).

Transient bombardments and gene expression analysis

One to 4 μ g of DNA were coated onto gold particles as described elsewhere (Frame, et al. 2000). Tissue was bombarded at 1100 psi, at a 6 cm distance from the macrocarriers. Plates were wrapped with Parafilm and incubated in the dark at 28°C for 3 d. For GUS analysis, samples from each plate were placed in a 6- or 24-well plate containing X-Gluc, sealed and incubated in the dark at 37°C before observation for GUS staining. For GFP analysis and GUS stained samples plates were visualized and imaged under using an Olympus SHZ10 stereoscope (Leeds Precision Instruments, Inc., Minneapolis, MN) coupled to a SPOT RT color CCD camera (Diagnostic Instrument Inc., Sterling Heights, MI). Images were taken under bright field for GUS stained samples or using a band pass exciter at 460 – 490 nm with emission filter 510 – 550 nm for GFP detection, and acquired using SPOT Advanced software.

Results

Establishment of a continuous endosperm culture

Three different genotypes of maize were used to test their abilities to initiate callus formation in isolated endosperms. Ears of B73, B104 and Hi II genotypes were harvested at 11 DAP, sterilized and dissected as described in Materials and Methods. Murashige and Skoog (MS) based medium with and without asparagine (MSA) were tested. From our experiments we observed that proliferous callus culture can only be obtained on the medium containing asparagine (MSA). Three months after the culture initiation, a few endosperms of Hi II genotype started to develop callus (Fig. 1A, arrow). Frequency of endosperm callus (ENC) culture initiation was zero for B73 and B104 genotypes, and about 3% (three of 100 isolated endosperm develop callus) for Hi II. Initial assessment of callus type was done by comparing with Hi II embryogenic callus (EMC) culture derived from immature embryos. When observing the two types of callus under the stereomicroscope, the endosperm callus did not have the friability that characterizes the Hi II embryogenic Type II callus, or the hardness that characterizes Type I callus. Calli growing on kernels were subcultured onto fresh MSA plates and allowed to grow for 1 to 2 months. Growth was slow under these conditions. Subculturing the callus every 10 days to two weeks proved ideal conditions to accelerate callus growth. Cultures have been stable and growing for four years under the conditions described.

Hi II maize endosperm callus cells differentiate into aleurone and starchy endosperm cells

To examine the nature of the endosperm callus culture compared to fresh kernels we fixed, embedded and sectioned the tissue for analysis by light microscopy. Sections were stained with toluidine blue O to study general morphology of the cells (Fig. 1D, E). Wild type Hi II kernel (Fig. 1D) dissected at 11 DAP shows the traditional staining of a maize kernel, with the pericarp (p), aleurone (a) and starchy endosperm (se) cell layers differentially stained by toluidine blue O. Aleurone cells show a densely stained cytoplasm representing a tightly packed single layer between the pericarp and starchy endosperm. On

the other hand, starchy endosperm cells show a lightly stained cytoplasm, with numerous starch granules developing in the cells (Fig. 1E). The ENC sections (Fig. 1E) show cells of densely stained cytoplasm (c) and nucleus (n), and also highly vacuolated (v) cells. Initial morphological analysis of the ENC indicates that the cells were more aleurone-like, than starchy endosperm-like.

To further study the physiology of the endosperm callus cells, freshly isolated endosperm tissue and ENC sections were stained using iodine to determine the presence of starch (Fig. 1F – I). When whole pieces of callus were stained with iodine solution, an EMC culture stained brown (Fig. 1C), while the ENC culture stained both brown and dark purple (Fig. 1B). Wild type 11 DAP Hi II kernel sections show purple I₂/KI-starch complexes in the granules (Fig. 1F, arrows) when viewed under bright field; the localization of starch granules corresponds to the starchy endosperm (Fig. 1G) when viewed under phase contrast. ENC sections, though presumably aleurone-like from toluidine blue O staining, showed some starch granule staining as well (Fig. 1H, arrows). This fact indicates that the cells in the endosperm callus culture are not exclusively aleurone or starchy endosperm, but are a mix of both.

Maize endosperm storage protein transcripts are detectable in Hi II maize endosperm callus tissue

We further investigated if the starchy endosperm cells present in the ENC culture retained starchy endosperm characteristics regarding protein expression. The endosperm tissue is not only the major organ for starch deposition in maize but it also serves as a protein storage reservoir. To determine the presence or absence of transcripts characteristic of maize endosperm tissue we carried out semi-quantitative PCR reaction (sqPCR) on cDNAs derived from maize 11 DAP developing endosperms (DE), ENC and EMC total RNAs (Fig. 2A). Zein storage proteins that express in starchy endosperm were chosen as main marker proteins. Also tested were constitutive protein transcripts such as actin and luminal binding protein as controls. Genes tested and primers used are listed in Table 1.

Fig. 2A shows the result for the semi-quantitative PCR (sqPCR) of zein and control transcripts. Constitutive transcripts for maize actin and maize luminal binding protein (BiP3)

showed equal intensities in all three tissues tested as expected. On the other hand, the transcript amplifications for most of the zein genes tested were differentially expressed in ENC, EMC and DE.

Transcripts were detected for 19 kD α -, 22 kD α - and 18 kD δ -zeins in DE, but in ENC and EMC the levels were very low or undetectable. From previous studies (Dolfini, et al. 1992; Woo, et al. 2001) it is known that transcript levels of 19 kD α - and 22 kD α -zeins peak at 17 DAP. At 10 DAP α -zein transcripts are of low abundance and restricted in localization to certain areas of the endosperm. Since we do not know which areas of the dissected endosperms proliferate into callus, this could be an explanation as to why levels of α -zeins in ENC are much lower than those observed in DE.

On the other hand, transcripts were detected in both ENC and DE samples for 10 kD δ -, 16 kD γ -, 27 kD γ - and 50 kD γ -zein. It has been shown that 16 kD γ - and 27 kD γ -zeins are expressed strongly and uniformly throughout the starchy endosperm (Woo, et al. 2001) and could be a consequence of their role in protein body assembly. The 10 kD δ -zein, however, occurs usually at lower levels compared to the other zeins (Woo, et al. 2001). It has been shown that accumulation of 10 kD δ -zein transcript is restricted to a small group of cells of the abgerminal side of the endosperm (Woo, et al. 2001). This observation may be helpful for understanding where some cells in our culture originated from.

Interestingly, transcripts for 16 kD γ - and 27 kD γ -zein were also detected in EMC. To determine whether the presence of these transcripts in EMC were the products of sample contamination, or contamination with DNA template, we conducted validation experiments. The strategy is schematically presented in Fig. B1. Primer set P1-P2 is targeted to amplify an 854 bp fragment spanning the promoter into the coding region of the 27 kD γ -zein gene, which will only give an amplification product in the presence of DNA template since promoter sequences are not transcribed. On the other hand, primer set P3-P4 was designed to amplify a 382 bp fragment in the coding sequence of 27 kD γ -zein (Fig. 2B1). Both primer sets were tested in total RNA, DNase I-treated-RNA and cDNA samples. Using total RNA template resulted in the presence of amplification bands of the sizes predicted (Fig. 2B2 and 2B3), which confirmed that the primer pair is functional. On DNase-treated RNA samples, we did not detect amplification for either set of primers, which proved that samples used for

cDNA synthesis were free of contaminating DNA. cDNA samples gave no PCR product for P1-P2 primers, ruling out the possibility of DNA contamination in the EMC. The fact that the same cDNA samples were used for all sqPCR reactions also rules out contamination with ENC or kernel RNA, where we would see the same patterns of amplification for all genes tested.

The sqPCR results show that the ENC culture possesses the ability to transcribe storage protein genes that are commonly expressed in the starchy endosperm of developing maize kernels. While the morphology analysis showed mixed populations of cells, the presence of zein transcript confirms that ENC has functional starchy endosperm cells as well, capable of transcribing maize storage protein genes.

Maize endosperm storage proteins are expressed and accumulate in Hi II maize endosperm callus tissue

Next, we wanted to confirm if ENC derived from Hi II genotype also accumulated certain zein proteins to detectable levels. Figure 3A shows the coomassie stain for aqueous and ethanol protein extracts from DE, ENC and EMC cultures. Zeins are extracted readily in ethanol based buffers, as can be seen in Fig. 3B and 3C. These blots show that ENC, unlike EMC, accumulate zein storage proteins, α - and γ -zeins to degrees comparable to those found in the fresh endosperms.

Similar findings had been reported for endosperm cultures of the inbred line A636 in which zeins were also detected (Shimamoto, et al. 1983). However, our experiments are able to identify two of the main classes of zeins, compared to the generic ‘zein’ antibody used previously (Shimamoto, et al. 1983). Figure 3 shows that more than one class of zein is accumulated (γ -zeins in Fig. 3B and α -zeins in Fig. 3C). The ability of the Hi II ENC culture to transcribe, translate and accumulate zein storage proteins can be maintained over the course of four years on solid medium.

Characterization of starch metabolism enzyme activity

Due to the nature of the endosperm tissue, accumulation and activity of starch biosynthetic enzymes are also expected. To determine whether starch enzymes were active

in ENC maintained over a course of three years, we carried on zymogram analysis based on reported protocols (Dinges, et al. 2001). Proteins from ENC, EMC and DE were separated by native-PAGE on a starch-containing gel and visualized by iodine staining (Fig. 4). Iodine will form an insoluble purple precipitate in presence of starch. Upon hydrolysis, highly branched regions will stain red, while regions with less branching will stain blue. Complete starch hydrolysis can be observed as white bands in the purple background, where the iodide cannot bind.

Figure 4 displays activities of several starch metabolism enzymes. The gel was compared to the published results (Colleoni, et al. 2003) in which starch-metabolizing activities were characterized. It is notable that a similar set of enzymes is present and active in EMC, ENC and DE samples. ENC shows activities of branching enzyme I, pullulanase and α -amylase. However, branching enzyme II and isoamylase activities are hard to discern. Identifying active starch metabolism and the enzymes doing the work opens possibilities to further study the starch synthesis in ENC.

Contrary to α -amylase, β -amylase activity is only detectable in EMC and ENC, but not in DE. This observation, together with the fact that α - and β -amylase activities seem stronger in both in EMC and ENC, may suggest a potential response to tissue culture conditions such as the high sucrose content in the culture medium. Identification of starch metabolism activities in ENC opens a window for future characterization of starch, starch properties of ENC compared to kernel counterparts, and starch metabolism enzyme response to *in vitro* culture.

Endosperm callus culture for transient analysis of gene expression

One of the motivations for establishing an ENC is to use it for rapid gene testing, avoiding the lengthy process required for developing stable transgenic maize plants or requirement to isolate fresh endosperm material. To test if our ENC is suitable for transient gene expression analysis, we used particle bombardment to deliver a series of constructs including reporter genes GUS and GFP under control of different promoters. Marker genes driven by constitutive promoters maize Ubiquitin 1 (PUBi) and CaMV 35S (P35S) resulted in detectable expression of both GUS enzyme (Fig. 5A) and GFP protein (Fig. 5B). The

aleurone specific promoter Viviparous 1 (PVp1) also resulted in detectable accumulation of GUS enzyme (Fig. 5C).

For starchy endosperm specific promoter evaluation, the GFP expression could be easily detected in ENC bombarded with the construct carrying the maize 27 kD γ -zein promoter (Fig. 5D). Interestingly, no expression was observed in bombarded ENC tissue with 22 kD α -zein (Fig. 5E) or 27 kD γ -zein driven GUS (data not shown).

Compared to a typical transient gene expression assay using GUS or GFP as marker using particle bombardment in maize embryo-derived callus tissue, the ENC transient results were not very informative due to low and sporadic expression. Expression of constitutive PUb1 and P35S, as well as PVp1 highlights the presence of aleurone-like cells in the culture, a property that has been used recently to study the position effect on aleurone cell differentiation (Gruis, et al. 2006). We cannot explain why we did not see more rigorous marker gene expression under these endosperm-specific promoters in the ENC culture. However, this same phenomenon occurs when bombarding endosperm slices of developing endosperms (data not shown) and has been reported for other endosperm suspension cultures (Quayle, et al. 1991). When stable transgenic maize lines carrying the P27 γ z -GUS and the P35S-GUS were analyzed, the strong GUS expression could be observed in the seed endosperm for the P27 γ z (Fig. 5G), but predominantly in seed embryo and aleurone layer for the 35S P (Fig. 5H).

Unsuccessful expression of a P22 α z-driven GUS was an unexpected result considering that α -zein proteins had been detected in ENC (Fig. 3C). One explanation is that additional factors are required for achieving transient gene expression when using α -zein promoter. It is known that maize Opaque 2 (O2; (Schmidt, et al. 1992) and prolamin box binding factor (PBF; (Vicente-Carbajosa, et al. 1997) proteins act as transcription factors for α -zein protein expression. When maize O2 and PBF were co-bombarded with the GUS gene driven by rice seed storage protein glutelin promoter (*GtI*), the transient GUS expression was enhanced 3 to 6 folds compared to the *GtI*-GUS control in rice immature endosperm cells (Hwang, et al. 2004).

Another explanation is that the 22 kD α -zein does not express strongly at this particular developmental stage of the endosperm. Our sqPCR results showed that 22 kD α -zein

transcripts are at very low levels (Fig. 2). Future experiments co-delivering the transcription factors could help elucidate if the α -zeins in the ENC could be increased by more transcripts of O2 and PBF.

Endosperm culture for stable transgene analysis

Fig. 6 demonstrates the application of ENC for stable gene expression analysis in tissue specific manner. A ENC culture was established from a transgenic Hi II maize that expresses a translational fusion between a *E. coli* B subunit of the heat labile enterotoxin (LT-B) and the green fluorescent protein (GFP) under the control of the maize seed specific 27 kD γ -zein promoter. The fusion protein accumulates in the secretory pathway of transgenic maize cells of developing kernels (Moeller, et al. 2009). To determine if the fusion protein remained functional in ENC *in vitro*, we compared the fusion gene expression in both ENC and DE under the confocal microscopy.

As can be seen in Fig 6, green fluorescence is detectable in both tissues, as expected coming from fluorescent kernels of a transgenic ear. The detection of green fluorescence confirms that GFP is still active in the cultured endosperm cells. Samples were counter-stained using propidium iodide for nucleic acid binding. In this experiment, the dye successfully stained nuclei in developing endosperm (Fig. 6B) but did not penetrate the cultured endosperm cells as easily, resulting in propidium iodide signal elsewhere in the cell (Fig. 6E). On the other hand, the immature embryos isolated from the GFP-expressing seeds did not show any green fluorescence (data not shown), indicating that the fusion protein LT-B::GFP under P27 γ z expresses in a tissue specific fashion.

The successful identification of GFP in transgenic ENC emphasizes the potential utilization of ENC cultures for transgene analysis expression. The system can potentially be used to study protein folding, processing and subcellular localization. Recently, an endosperm callus culture using transgenic material was used to study cell fate specification (Gruis, et al. 2006).

Discussion

This work describes the establishment and maintenance of a continuous *in vitro* callus culture derived from 11 DAP freshly isolated endosperm of genotype Hi II over a period of four years. Initiation of the ENC for Hi II was relatively straightforward following the published medium used to establish ENC for inbred A636 (Ueda and Messing 1991), although the frequency was low (avg 3%). We were unable to initiate ENC for inbred B73 and B104.

Gruis, et al (2006) used ENC from stable transgenic Hi II lines expressing marker proteins under various tissue specific promoters to study endosperm cell fate. The main differences in culture medium are that they used 15% sucrose (compared to 3% in this work), 400 mg/L asparagine (compared to 2 g/L in this work) and 5 mg/L thiamine (0.5 mg/L in this work). They also added 10 μ g/L 6-benzylaminopurine (Gruis, et al. 2006). For the initiation of the ENC, they used fresh endosperm at 6 DAP instead of 11 DAP used in this work.

Similar to Gruis, et al (2006), the morphological characterization of the ENC in our work confirmed the dual nature of the culture, revealing presence of both aleurone-like and starchy endosperm-like cells. Many reported studies using ENC cultures were in the form of suspension culture (Felker 1987; Felker and Goodwin 1988; Quayle, et al. 1991; Riedell and Miernyk 1988; Shannon and Liu 1977; Ueda and Messing 1991). The ENC established in our work is a continuous callus culture on Petri plate that is still viable after four years *in vitro*. Compared to suspension culture, plate culture is easier to manage, requiring less frequent transfer, less medium and space for storage, and no need for dedicated equipment such as a shaker incubator.

In our study, we performed transcript analysis including members of the α -, δ -, and γ -zeins. Results for transcript analysis were in general terms, as expected and similar to those reported (Ueda and Messing 1991). Identification of transcripts for the 10kD δ -, 16kD γ -, 27kD γ -, and 50 kD γ -zeins in a long-term culture derived from endosperms (11 DAP) corresponds with the patterns of expression for these types of zeins (Woo, et al. 2001). Very low to undetectable levels of transcripts of the 19kD α -, 22kD α -, and 18 kD δ -zeins in ENC cultures correlates well with what has been observed previously for developing endosperms (Woo, et al. 2001) where at 10 DAP transcripts are not only of low abundance, but limited to

specific regions of the endosperm tissue. Our results are in line with what was observed for transcript analysis of 10kD δ -, 15kD β - and 27kD γ -zeins in endosperm suspension cultures of line A636 started at 13 DAP (Ueda and Messing 1991), specifically for 10kD δ - and 27kD γ -zeins. Ueda and Messing (1991) did relative quantification of the transcripts and showed that transcripts for 10kD δ -, 15kD β - and 27kD γ -zeins showed reduced levels by 199-, 22-, and 46- fold, respectively, when compared to developing endosperms (16 DAP). Our results with semi-quantitative PCR confirm what was observed for 10kD δ - and 27kD γ -zeins, and suggest that the same is especially true for α -zeins. Massively parallel signature sequencing (MPSS) was used to compare the steady-state levels of endosperm marker transcripts between *in vitro*- and in planta-grown endosperms (Gruis, et al. 2006). Genes analyzed included the 27kD γ - and 16kD γ -zeins and two highly aleurone-specific genes. Their results indicated that when cultured *in vitro*, endosperm 27kD γ - and 16kD γ -zein transcript levels initially increase, but markedly decrease after a period in culture. No long term culture data is reported (Gruis, et al. 2006). Interesting prospects include future studies of the induction or repression of these genes *in vitro*, and the significance that may have biologically.

At the protein level, several groups had probed to detect zeins (Racchi and Manzocchi 1988; Shimamoto, et al. 1983) in the past. Maize 19 kD and 22kD α -zeins were detected in ethanol extracts and protein bodies recovered from A636 endosperm suspension cultures; zein levels from A636 ENC were substantially lower than from 20 DAP kernels (Shimamoto, et al. 1983). Zeins were also detected in pigmented and unpigmented long term cultures of maize hybrid K55/W23 at 10 DAP, even though they were not detected in freshly isolated developing endosperms of the same age. Both of the afore-mentioned studies used antibodies that reacted to 19 kD and 22 kD zein bands, likely the α -zeins of these molecular weights. We have used two antibodies that react specifically to α - and γ -zeins. Our Western blots show that the accumulation of γ -zeins corresponds to transcript level accumulation. However, for α -zeins the presence of proteins was somewhat unexpected, since transcript levels were very low. This could be the result of the endosperms ‘aging’ *in vitro* to later stages of development, as has been suggested previously (Shimamoto, et al. 1983). Protein accumulation could also be better tolerated by the culture than transcript accumulation. This

area presents challenges to the researcher in terms of predictability of the culture for future experiment design.

The presence of 16 kD and 27 kD γ -zeins in EMC culture was not expected. It is possible that there are some low expressions of these zein genes in embryo tissues at early stage of seed development. In fact, we have observed some “leaky” expression of a color marker gene in stable transgenic embryo culture while using the 27 kD γ -zein promoter (K. Wang, unpublished).

Morphological analysis of ENC of genotype A636 has shown that suspension cultures can accumulate starch (Felker 1987). In this work we confirm that Hi II ENC cultured in plates also accumulates starch, as evidenced by dark purple staining with iodide. We also present evidence that starch metabolism enzymes are active by using a starch zymogram analysis. Patterns of enzyme activities of EMC and DE are similar to ENC and to each other. However, EMC and ENC showed strong activities of putative α - and β -amylases compared to DE. Future work will help establish if this is the result of *in vitro* culture and what role these enzymes are playing in these cells.

Our initial interest of establishing an ENC was to perform transient expression analysis of transgenes driven by starchy endosperm-specific promoters to reduce the dependence on generating stable transgenic lines as well as preparing fresh developing endosperm materials. However, this work suggests that bombardment of ENC for transient analysis using reporter genes as we typically do for a similar transient assay in the EMC system may not be the best approach at this stage. The successful expression of reporter genes driven by constitutive promoters in ENC reflects the presence of aleurone-like cells in the culture. However, the level of expression obtained is much less than that obtained by similar analysis using other systems such as EMC, immature zygotic embryos or even leaves. Bombardment of reporter genes under control of endosperm-specific zein genes failed to generate any transient expression, with the exception of P27 γ z-GFP in our work. This observation had been reported previously in A636 ENC system (Quayle, et al. 1991). These authors reported failure to express zein-promoter driven constructs and used an attenuated 35S promoter adding a “-300 box” from zeins, which specifically enhanced transient expression in ENC but

not EMC. In our hands, freshly isolated developing endosperm performed similarly to ENC culture in transient analysis (data not shown).

The major advantage of having a Hi II ENC at this point is for analysis of transgene expression in endosperm. Because of the ease of ENC establishment for a transformation amenable genotype and longevity of the culture, it is then possible to establish callus cultures for transgenic tissue as well. In this work we show that the green fluorescent protein remained active in ENC. In a similar fashion, Gruis, et al (2006) established an ENC from 6 DAP kernels of transgenic Hi II maize carrying endosperm specific expressing fluorescent proteins (RFP, YFP). The low sugar and high asparagine in our medium, compared to high sugar and low asparagine in Gruis et al (2006) might have some effect in the long term survival of our cultures versus theirs.

While an ENC culture cannot be solely used for the interpretation of gene functions and regulations, it can provide a tool for testing hypotheses related to mechanisms underlying endosperm development and protein storage deposition in a fast, flexible and cost effective way, as long as proper controls are included to rule out any *in vitro* tissue culture induced variations.

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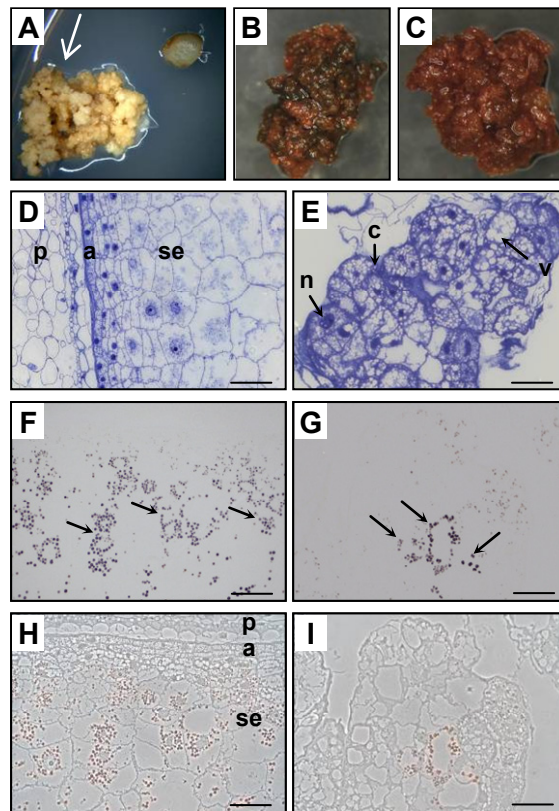


Figure 1 Maize endosperm callus culture derived from Hi II developing endosperms. **A**, Developing Hi II maize endosperms dissected at 11 DAP are cultured in MSA medium for callus proliferation in some endosperms (white arrow). **B**, Hi II maize endosperm callus stained with I2/KI. **C**, Hi II maize embryo-derived callus stained with I2/KI. **D-I**, Semi-thin sections (1µm) of developing Hi II maize endosperms (**D**, **F**, **H**) and maize endosperm callus (**E**, **G**, **I**) were stained with toluidine blue (**D**, **E**) and I2/KI (**F** - **I**). Stained sections were viewed and imaged under brightfield (**D** - **G**) and phase contrast (**H**, **I**). Bars = 20 µm. P, pericarp. A, aleurone. Se, starchy endosperm. N, nucleus. C, cytoplasm. Black arrows, starch granules.

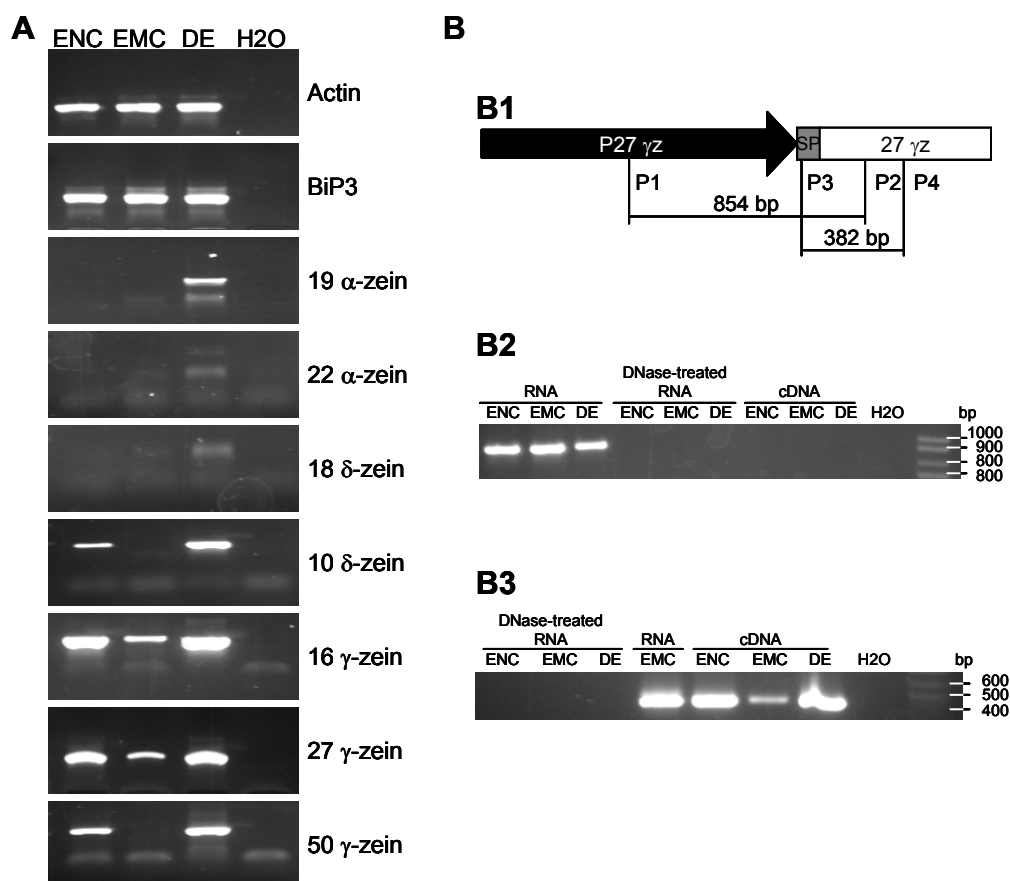


Figure 2 Semiquantitative PCR on cDNA from maize endosperm callus culture, maize embryo callus culture and maize kernels 11 DAP. **A**, Maize storage proteins transcripts. **B**, Analysis of 27 kD γ -zein (**B1**) expression using primers to determine possible template contamination in embryo callus samples. **B2**, Amplification with primers spanning the promoter region and coding sequence. **B3**, Amplification with primers spanning the coding region only. DE, developing maize endosperms (11 DAP). ENC, maize endosperm callus culture. EMC, maize embryo callus culture.

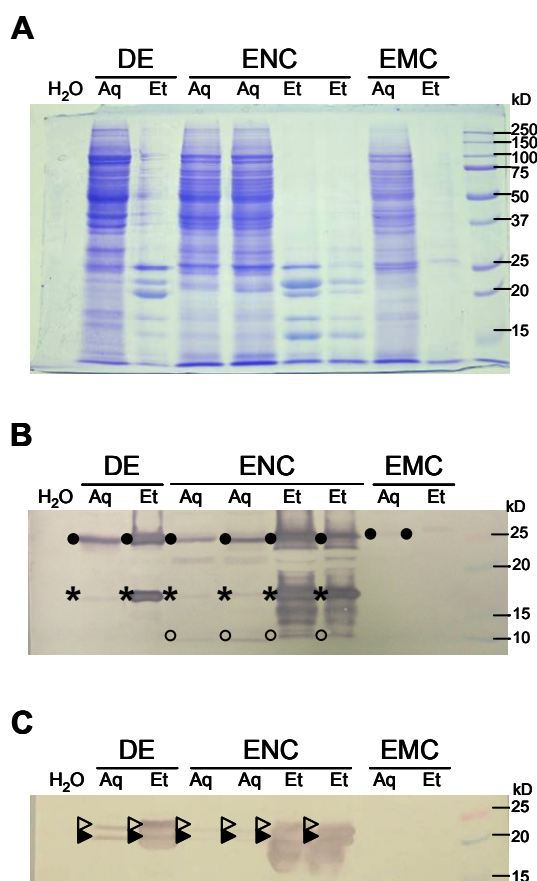


Figure 3 Proteins from fresh endosperm (11DAP), endosperm callus and embryo callus. Total proteins extracted with an aqueous (Aq) and ethanol (Et) buffers were separated by SDS-PAGE and stained for total proteins with coomassie stain (**A**). Separated proteins were transferred onto a nitrocellulose membrane and probed using anti γ -zein (**B**) or anti α -zein (**C**) antibodies. DE, developing maize endosperms (11 DAP). ENC, maize endosperm callus culture. EMC, maize embryo callus culture. Closed circle, 27 kD γ -zein. Asterisks, 16 kD γ -zein. Open circle, 10 kD γ -zein. Open arrowhead, 22 kD α -zein. Closed arrowhead, 19 kD α -zein.

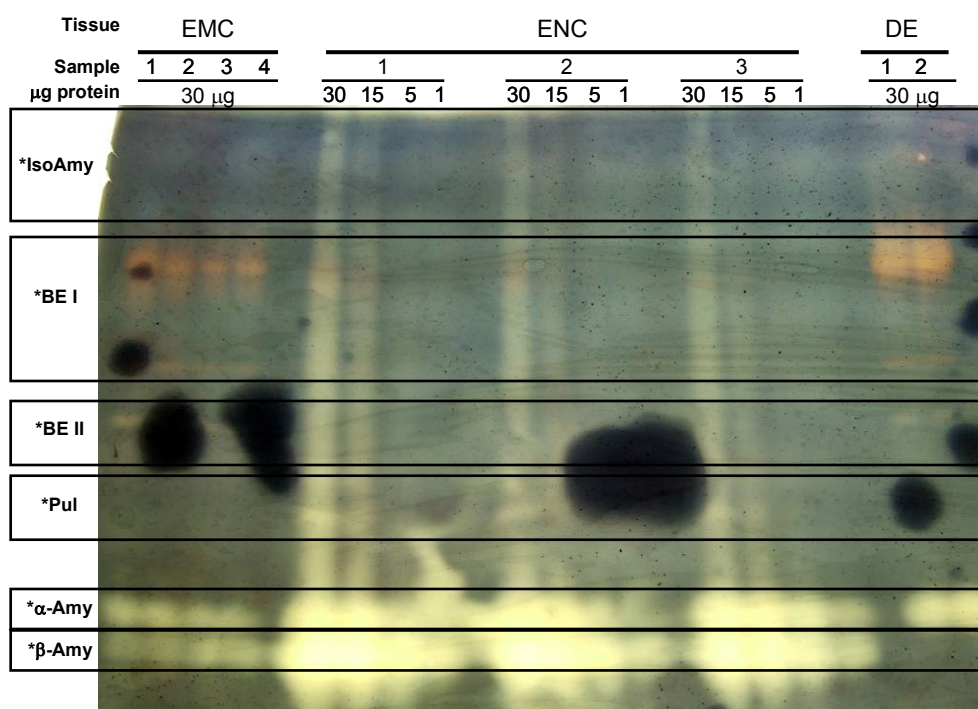


Figure 4. Zymogram analysis of endosperm callus culture, embryo callus culture and developing maize endosperms for detection of starch enzyme activities. Aqueous protein extracts were separated on Native-PAGE gels containing maize starch. Activity bands were revealed by incubation of the gel in I2/KI solution and washing in water. White and red bands indicate regions where enzymes have degraded the starch substrate. DE, developing maize endosperms (11 DAP). ENC, maize endosperm callus culture. EMC, maize embryo callus culture. E, empty lanes. *, Putative starch metabolism enzymes based on (Colleoni *et al.*, 2003). Dark spots are due to unsolubilized starch in the gel stained purple with iodide. IsoAmy, iso-amylase. BEI, branching enzyme I. BEII, branching enzyme II. Pul, pullulanase I. α -Amy, α -amylase. β -Amy, β -amylase.

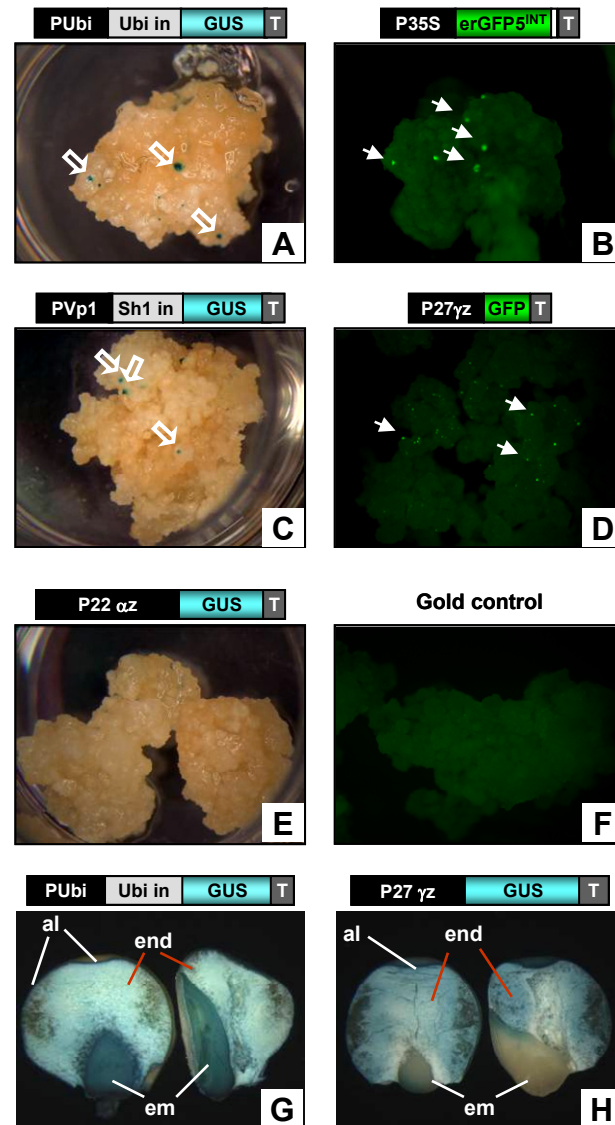


Figure 5 Analysis of transient gene expression in bombarded endosperm callus. Maize endosperm callus was bombarded using the indicated constructs. GUS and GFP expression were assessed visually. **A**, PUb1-GUS (Christensen and Quail, 1996). **B**, P35S-GFP (Mankin and Thompson, 2001). **C**, PVp1-GUS (Cao et al, 2007). **D**, P27 γ z-GFP (Shepherd and Scott, 2008). **E**, P22 α z-GUS. **F**, Gold control. **G**, Transgenic maize kernel expressing pUb1-GUS in embryo tissue and aleurone. **H**, Transgenic maize kernel expressing p27 γ z-GUS (Chikwamba, unpublished) in the starchy endosperm. Block arrows, GUS foci. Arrows, GFP foci. GUS, β -glucuronidase. GFP, green fluorescent protein. PUb1, maize ubiquitin 1 promoter. P35S, cauliflower mosaic virus 35S promoter. PVp1, maize viviparous 1 promoter. P27 γ z, maize 27 kD γ -zein promoter. P22 α z, maize 22 kD α -zein promoter. Ubi in, ubiquitin intron. Sh1 in, shrunken 1 intron. erGFP5^{INT}, GFP targeted to the endoplasmic reticulum. Al, aleurone. End, endosperm. Em, embryo.

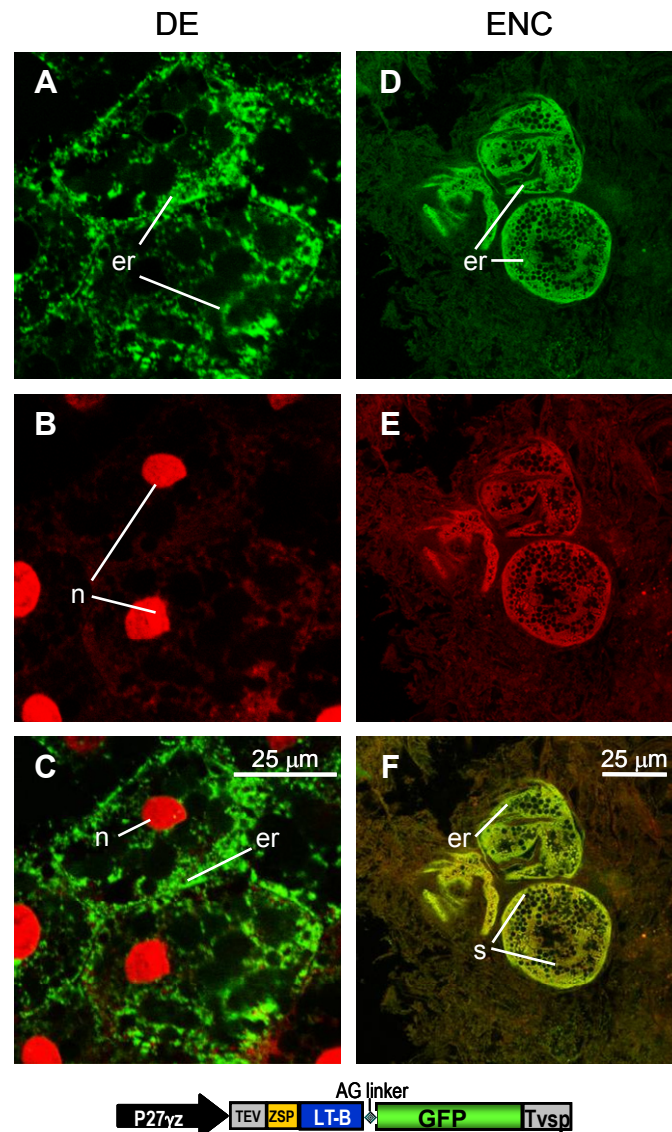


Figure 6. Analysis of transgene expression in developing endosperm and endosperm callus. A transgenic maize line expressing the B subunit of the heat labile enterotoxin (LT-B) of *E. coli* fused to the green fluorescent protein (GFP) was used as a source of transgenic endosperm at 11 DAP. Developing endosperms (DE) were imaged (A-C) or plated for callus induction. Two months after culture initiation, callus induced tissue (ENC) was visualized (D-F). Samples were screened for GFP expression (A, D) and propidium iodide counterstain (B, E). Separate channels were merged electronically (C, F). Er, endoplasmic reticulum. N, nucleus. S, starch granules. P27 γ z, maize 27 kD γ -zein promoter. TEV, tobacco etch virus translational enhancer. ZSP, maize 27 kD γ -zein signal peptide. AG linker, alanine-glycine linker. Tvsp, vegetative soybean storage protein terminator.

Table 1

Primer sequences used for semi-quantitative PCR

Protein		Sequence	
Actin	F, 5'	ATTCAGGTGATGGTGTGAGCCACAC	3'
	R, 5'	GCCACCGATCCAGACACTGTACTTCC	3'
Luminal binding protein, cBiPe3	F, 5'	CGTTCCTGCTCGGCGTCCTGCT	3'
	R, 5'	CCACCCTTCTTGTCCAAACCATAGGC	3'
19kD alpha zein (D1, D2)	F, 5'	ATTCCACAATGCTCACAACAATACC	3'
	R, 5'	CAATAAGGTGGTAGGATTCGTCAAAGC	3'
22kD alpha zein (1, 3, 4, 5)	F, 5'	TCATTATTCCACARTGCTCACTTGCTC	3'
	R, 5'	CGCTGTTGTAGGTACGCAGCAGAGT	3'
18kD delta zein	F, 5'	ATGGCAGCCAAGATGTTTG	3'
	R, 5'	GCACTGTCATCATGTTTGGC	3'
10kD delta zein	F, 5'	ATGGCAGCCAAGATGCTT	3'
	R, 5'	CGCAGTGACATTGTGGCA	3'
16kD gamma zein	F, 5'	ATGAAGGTGCTGATCGTTGC	3'
	R, 5'	GCAGGACCACACCGTATGTC	3'
27kD gamma zein	F, 5'	ATGAGGGTGTTGCTCGTTGC	3'
	R, 5'	ATGCCTCAGGAACCTCGACGC	3'
50kD gamma zein	F, 5'	ATGAAGCTGGTGCTTGTGG	3'
	R, 5'	TGACCCTGGAATTGTTGTTG	3'

**CHAPTER 6: STANDARD METHOD FOR MEASUREMENT OF
ENDOGLUCANASE ACTIVITY IS NOT ADEQUATE FOR
CHARACTERIZATION OF MAIZE-DERIVED ENDO-1,4- β -
GLUCANASE FROM *RUMINOCOCCUS ALBUS***

Lorena Moeller, Qinglei Gan, Hongxin Jiang, Mahwish Hafeez, and Jay-lin Jane and Kan
Wang

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Abstract

We have generated two lines of transgenic corn that express a maize codon-optimized endo-1,4- β -glucanase (mEgl) from *Ruminococcus albus* under constitutive or seed-specific expression. Stable insertion of the transgene was determined using Southern blotting. Constitutive activity of the mEGI enzyme was detected using a carboxymethylcellulose (CMC)-zymmogram assay. A modified CMC microplate assay was used to quantify activity in transgenic maize kernels of line P199. Gel permeation analysis of CMC and enzyme-treated CMC revealed that the transgenic protein extracts have the ability to hydrolyze CMC to different degrees. The temperature profile of the enzyme extracts using CMC assay and high levels of CMC-activity in non-glucanase maize lines suggested that the CMC method is not appropriate for characterization of recombinant glucanase activity in transgenic corn. A fluorescent assay (MUCase) based on the hydrolysis of 4-methylumbelliferyl β -cellobioside and detection of fluorescent 4-methylumbelliferone was also tested. Detection of high MUCase activity in non-glucanase lines confirmed that MUCase assay is not appropriate for characterization of recombinant glucanase produced in maize either. The potential applications of maize as a biofactory for cellulase enzyme production highlight the importance of developing an adequate method for characterization of transgenic lines and proper interpretation of future utility in the biofuel industry.

Introduction

Cellulose is the most abundant polymer on Earth and an important renewable resource that can help meet the continuously increasing demand for energy worldwide. For this reason, the concept of using cellulosic material for biofuel production is greatly supported by governments in industrialized countries [1]. Genetic engineering of plants for enhanced biofuel production presents ample opportunities to enhance the utilization of cellulosic biomass and reduce the costs of conversion to biofuels. The topic is of great interest nowadays and has been reviewed extensively [2, 3]. As has been described for maize [4], biotechnology presents opportunities to improve plant cellulosic biomass conversion to ethanol via different approaches [2, 3]. Plants can be engineered to produce more biomass, and hence, augment the starting material for biofuel production [5, 6]. Lignin modification could allow for higher accessibility of cellulose-degrading enzymes to access cellulose and improve yields of bioprocessing [3, 7]. Finally, plants can be engineered to produce cellulolytic enzymes in the plant's biomass in order to reduce the cost of the overall process [8].

The enzymatic hydrolysis of cellulose involves action of at least three enzymes in concert: endo-1,4- β -glucanase (E.C.3.2.1.4), exo-cellobiohydrolase (E.C.3.2.1.91), and β -glucosidase (E.C.3.2.21) [9]. Due to its important role in initial cellulose deconstruction, several groups have focused on using endo-1,4- β -glucanases from different hosts as a target for heterologous expression in tobacco [10, 11], potato [12], *Arabidopsis* [13], barley [14], rice [15] and maize [16, 17]. Endoglucanases are widely spread across most kingdoms, but many bacterial and fungal systems possess highly efficient cellulolytic enzyme clusters that include powerful endoglucanases. Glucanases expressed in plants have, therefore, been isolated from different bacteria and fungi such as from *Ruminococcus albus* F-40 [10], *Acidothermus cellulolyticus* [12, 13, 15, 17] and *Trichoderma reesei* [14]. The successful expression of these enzymes in plants often depends on detailed planning and design. Gene optimization for preferred codon usage often results in higher expression of the heterologous enzyme in the plant systems [18]. Choice of promoter sequences is critical for tissue and developmental stage expression of the recombinant protein. The cauliflower mosaic virus (CaMV) 35S promoter has been used for constitutive expression [10, 15], while other

promoters such as the hybrid hi-pI α -amylase promoter [14], the Rubisco small subunit RbcS-3C promoter [12] and Mac promoter [12] have been used for expression in specific tissues. Subcellular targeting can also affect the accumulation of recombinant cellulases in plants [11] and hence, several groups have tested targeting to different cellular compartments such as the cytosol [11], chloroplast [11, 12], apoplast [11, 12, 15] and vacuole [12]. In general, observations suggest that accumulation in a cellular compartment other than the cytosol is favorable for enzyme accumulation [3, 11]. Due to its key role in secretory pathway protein folding and assembly, the ER is considered an ideal organelle for accumulation and storage of recombinant proteins in plants [19].

Characterization of recombinant endoglucanase activity is based on the principle of the enzyme's ability to digest β -glucan polymers such as CMC, Avicel, and plant-derived cell walls. Certain polysaccharides, including β -glucan, will form complexes with certain dyes, which render them useful in determining β -glucanase activity [20]. One such dye, congo red has been shown to bind covalently to β -glucan, O-(carboxymethyl)cellulose and O-(carboxymethyl)pachyman forming a red complex. However, regions where hydrolysis has occurred due to enzyme activity appear as cleared zones with area proportional to the $\log[\text{enzyme}]$ [20]. This method or a modification of it has been used repeatedly to study β -glucanase activity in protein extracts from transgenic material expressing recombinant cellulases [14, 21, 22]. CMC has been used as a standard substrate for measuring endoglucanase activity since the Commission on Biotechnology of International Union of Pure and Applied Chemistry (IUPAC) proposed a number of standard procedures for the measurement of endo-1,4- β -glucanase activities in 1984 [23]. A microplate-based carboxymethylcellulose assay [24] with determination of reducing sugars using dinitrosalicylic acid [25] was developed in order to make the standard method more high-throughput. Another method used to characterize transgenic lines expressing recombinant endoglucanase activity is based on the hydrolysis of methylumbelliferyl β -cellobioside (MUC) and detection of released fluorescent 4-methylumbelliferone (MU) [11, 12, 15-17]. Even though these two methods have been used to report endoglucanase activity in transgenic maize, the adequacy and specificity of the assays has not been addressed or studied in detail.

In this project we have codon optimized an endo-1,4- β -glucanase gene (Genbank accession number: M30928) from *Ruminococcus albus* for expression in maize under the control of CaMV 35S and the maize 27 kD γ -zein promoters for constitutive and seed-specific expression, respectively. The bacterial signal peptide has been replaced by a 27 kD γ -zein signal peptide for targeting to the ER, and an ER-retention motif (KDEL) has been added to the carboxy terminus to generate a modified endo-1,4- β -glucanase gene (mEgI). Southern blot analysis of T1 plants confirmed successful insertion and inheritance of the transgene in maize. Initial screening of transgenic maize callus showed that certain lines exhibited higher CMC-degradation ability than non-transgenic callus. CMC activity was not detected in protein extracts from dry kernels. We present the work done in order to further characterize the transgenic material carrying mEgI and the problems found when using the standard methods for measuring endoglucanase activity.

Materials and methods

DNA constructs and plant transformation

The endo-1,4- β -glucanase (EgI) gene sequence from *Ruminococcus albus* was obtained directly from Genbank, accession number M30928 [22] and further designed for maize expression *in silico*. The coding sequence was then codon optimized according to the maize codon preference. Codons with less than two thirds of the frequency of the most frequent codon were changed to the most abundant codon. Codons with two thirds or more of the frequency of the most frequent codon were kept in their original form in order to conserve codon diversity. The initial 43 aminoacids of the EgI protein were replaced by the maize 27kD γ -zein signal peptide in order to direct the protein to the ER. The amino acids KDEL, an ER retention motif, were added in the 3' end of the protein. The resulting coding sequence for the modified EgI gene (mEgI) was synthesized by The Midland Certified Reagent Company (Midland, TX). The mEgI sequence was then cloned into pAHC25 [26] under the control of the maize ubiquitin 1 promoter (UbiP) and nopaline synthase (Nos) terminator and into pRC3 (Chikwamba, unpublished) under the control of the 27kD maize γ -

zein promoter (27gzP) and Nos terminator to generate transformation plasmids pWL-1 and pWL-2.

Transformation of maize plants with constructs pWL-1 and pWL-2 (Fig. 3) was achieved by using the biolistic-gun method [27], and transgenic plants were grown to maturity in the greenhouse following standard protocols [28].

Polymerase Chain Reaction (PCR) analysis

Leaf tissue of young T1 plants was used for genomic DNA extraction using a cetyltrimethylammoniumbromide (CTAB) protocol [29]. PCR analysis was used to identify plants carrying the mEgI gene. Three reactions were used for screening purposes using primer pairs from the promoter to mEgI and from mEgI to the terminator. Primers for line P198 were sense: 5'-CAGAGATGCTTTTGTTCGCTTGG-3', and antisense: 5'-CAGGTTGTTGTAGGCGCTCG-3'. Primers for line P199 were sense: 5'-CTTGTTTCGCTTACGTCTGGATC-3', and antisense: 5'-TGTCGTTGGTGTGGGAGGTG-3'. Primers GG-1 (5'-ACCTCCCACACCAACGACAC-3') and GG-2 (5'-CAGGGGATGTCGTACTIONTGGC-3') were used for both lines.

For studying correlation of enzyme activity with gene presence, DNA from leaf or embryo tissue was extracted in 400 µL extraction buffer [200 mM Tris-Cl (pH 7.5), 250 mM NaCl, 25 mM EDTA, and 0.5% SDS (w/v)], followed by consecutive extractions in equal volumes of phenol and chloroform:isoamyl alcohol (24:1, V/V). DNA was precipitated using isopropanol and was cleaned using consecutive washes of 70 and 95% ethanol. DNA was resuspended in sterile water. About 100 ng of DNA were used for PCR using oligonucleotide primers 5'-CTGACCTGGAGTGGTACTTC-3' (forward) and 5'-ATTAAATGTATAATTGCGGG-3' (reverse) a 300 bp fragment from the junction of the mEgI coding sequence and the Nos terminator.

Southern blot analysis

Leaf tissue of PCR-positive T1 plants was used for genomic DNA extraction using a cetyltrimethylammoniumbromide (CTAB) protocol [29]. Ten to 15 µg of leaf genomic DNA was digested with restriction enzyme SacI at 37°C for at least 16 h and separated on a 1% agarose gel. Linearized DNA gel blot analysis [30] was conducted on DNA samples

with a ^{32}P -labeled glucanase PCR fragment amplified and purified from plasmids pWL-1 or pWL-2 using primer pairs GG-1 and GG-2.

Total soluble protein extraction, quantification and ammonium sulfate precipitation

Protein was extracted from ground tissue (leaves, roots, endosperm or embryo) in 500 μL of grinding buffer (50mM sodium acetate pH=5.5, 100 mM NaCl, 10% v/v glycerol, 0.5 mM EDTA, 1 mg/L leupeptin, 1.2 mg/L pefablock). The samples were centrifuged for 10 min and the supernatant was used as the total aqueous soluble extractable protein (TAEP). Extracts were stored at 4°C until needed. Total soluble protein concentrations were determined by the Bradford Assay [31] using bovine serum albumin (BSA) as a standard.

Protein precipitation was carried on as in [15]. Briefly, 771 μL of saturated ammonium sulfate was added to 400 μL of TAEP for a final ammonium sulfate concentration of 2.7 M achieving ~70% saturation at 4°C. Tubes were incubated on ice for 30 min, and then were centrifuged at 15,000 x g at 4°C for 10 min to pellet out protein. Pellets were resuspended in 200 μL protein grinding buffer.

Carboxymethylcellulose (CMC) zymogram analysis

A modified zymogram assay based on [21] was used. Briefly, substrate containing plates were prepared (0.5% CMC, 1% agar medium in 10 mM sodium phosphate buffer, pH 6.8) and allowed to solidify. Wells 4 mm in diameter were bore in the gel in order to fit samples and controls. Crushed callus samples or TAEP was added to the wells and gels were incubated for 18-20 h in a humid chamber at 28°C. Congo red (1mg/mL) aqueous solution was added to cover gel and let stand for 20 minutes. Sodium chloride 1.0 M was added next after a rinse with distilled-deionized water, and allowed to stand for 20 minutes. Clear halos appeared on a red background where hydrolysis of CMC had occurred.

Western blot analysis

An aliquot containing 50–100 μg of total aqueous extractable protein (TAEP) from maize kernels were boiled for 5 min and loaded onto a 15% polyacrylamide SDS-PAGE (Laemmli, 1970). The separated proteins were transferred to a 0.45 μm nitrocellulose membrane using the BioRad Semidry Transblot apparatus according to the manufacturer's instructions.

Membranes were incubated for 1 h at 25°C unless otherwise specified. Membranes were blocked with 5% DM in PBST (Phosphate Buffered Saline - 0.05% Tween-20 (v/v)). Primary antibody was a rat monoclonal anti-KDEL antibody [MAC 256] (ab50601; ABCAM INC., Cambridge, MA, USA) used at a dilution of 1:500 in 1% DM in PBST. Secondary antibody was a rabbit polyclonal anti-Rat IgG antibody (ab6734; ABCAM INC., Cambridge, MA, USA) used at a 1:1000 dilution in PBST. Colored bands were revealed by incubation with horseradish peroxidase substrate, 3,3',5,5'-Tetramethylbenzidine (T0565, Sigma, St. Louis, MO, USA).

Measurement of glucanase activity using carboxymethylcellulose (CMC)

A modified version of the carboxymethylcellulose – dinitrosalicylic acid assay for reducing sugars [24] was used to measure glucanase activity. Glucose standards of 0.01 M, 0.005 M, 0.0033 M, 0.0025 M, 0.002 M, 0.0013 M, and 0.001 M in 50 mM NaAc buffer, pH 4.8 were used in every assay to generate a calibration curve. Briefly, 50 µL of the protein samples were added to 50 µL of 1% carboxymethylcellulose (CMC) in 50 mM NaAc buffer and incubated at 50°C for 30min. The reaction was stopped using 100 µL DNS reagent (1.4% dinitrosalicylic acid, 0.28% phenol, 0.07% sodium sulfite, 28% Na-K-tartrate, and 1.4% NaOH) and the plate was incubated at 95°C for 5 min. The absorbances were measured at 540 nm in a spectrophotometer using 50 µL protein grinding buffer as blanks. Samples and standards were run in duplicate and arranged in a randomized fashion. Non-transgenic samples of maize inbred B73 were used for baseline readings in every assay.

Degree of hydrolysis and sepharose gel permeation chromatography

A 1% CMC solution was incubated with commercial cellulase or TAEP extracts from P199 and B73 maize kernels, in acetate buffer (pH 4.8) at 50°C. Reducing sugars were measured using DNS method [25] and glucose as standard. The degree of hydrolysis was calculated as the amount of reducing sugars released from CMC divided by total weight of CMC. CMC (0.5mg/mL) and enzyme-hydrolyzed CMC (2mg/mL) with 10% hydrolysis were subsequently loaded onto a Sepharose CL-2B gel-permeation column following published procedures [32].

Measurement of glucanase activity using methylumbelliferyl cellobioside (MUC)

MUCase assay was carried on as described [11]. Briefly, 1-4 μL of sample or standards was added to 100 μL reaction buffer (50 mM sodium acetate pH5.5, 100 mM NaCl, 0.5 mM 4-methylumbelliferyl β -D-cellobioside) in a 96-well plate, sealed and incubated at 50°C for 30 min. One-hundred μL of stopping buffer (0.15 M glycine pH 10) were added to each well to terminate the reaction. Fluorescence measurements were read in a spectrofluorometer using excitation and emission wavelengths of 360 nm and 465 nm, respectively. Standards ranged from 4 to 160 pmol 4-methylumbelliferone (4-MUF).

Results and discussion

Codon optimization, gene design and plant transformation

Figure 1 presents an alignment of the original endo-1,4- β -glucanase (EgI) gene from *Ruminococcus albus* and the codon optimized version used in this study (mEgI). Highlighted in black can be seen the nucleotide substitutions made to favor codon usage in maize compared to the bacterium. It can also be seen that the amino acid sequence, AA, is conserved regardless of the nucleotide substitutions performed.

Figure 2 shows a schematic representation of the gene design strategy for generating mEgI. It can be seen that the N-terminal 57 amino acids from *Ruminococcus albus* EGI protein were removed and replaced by an 18-amino-acid sequence corresponding to the maize 27 kD γ -zein signal peptide to direct the mEgI to the ER lumen. At the C-terminus of the mEGI protein we also added a KDEL amino acid motif for retention of the recombinant glucanase in the ER. A final schematic representation of the constructs used in plant transformation is presented in Fig. 3A, where the mEgI-KDEL gene was placed under the control of the maize Ubiquitin 1 or 27 kD γ -zein promoters in plasmids pWL-1 and pWL-2, respectively. Successful gene integration and inheritance were shown by Southern blot analysis presented in Figs. 3B and 3C for transgenic lines P198 and P2199 generated by transformation with plasmids pWL-1 and pWL-2, respectively. It can be seen in Fig. 3B that transgene copy numbers ranged from two to nine copies for line P198. For line P199 we

cannot make a conclusive claim for all events tested, though information from Fig. 3B and 3C is enough to say that transgene copy numbers ranged from two to at least five.

CMC zymmogram analysis and Western blotting

Initial characterization of transgenic callus events of P198 was conducted by CMC zymmogram analysis. Inclusion of CMC in the media and incubation of TAEP from transgenic callus resulted in hydrolysis of CMC by endoglucanases. Areas of hydrolysis were visualized by staining the gel with congo red, a dye that binds CMC but not its hydrolyzed forms. Therefore, areas of hydrolysis are visualized as clear halos in a red background. Using commercial cellulases as positive control, and non-transgenic callus as negative control, it can be seen in Fig. 4A that some transgenic events (P198-3 and P198-8) showed higher CMC hydrolytic activity than non-transgenic extracts. This result was very encouraging suggesting that the transgene was not only integrated successfully in the plants' genome, but it rendered functional proteins *in vivo*.

Seeds from transgenic line P199 were also tested using the CMC zymmogram assay. However, extracts from dry seed showed no significant CMC hydrolytic activity when tested in the same manner as we tested the callus for the constitutive lines. In order to establish if the recombinant protein was present in kernels of line P199, we tried using an anti-KDEL antibody in western blot analysis, since the mEgI construct carries a KDEL sequence for retention in the ER. The antibody titer was very low, and results were encouraging but not conclusive. It can be seen in Fig. 4B that protein extracts of line P199 have an enhanced band at ~50 kD compared to B73 non-transgenic extracts. However, due to the high background and the high expense of the antibody we did not pursue further characterization using Western blotting.

Microplate based endoglucanase activity based on CMC hydrolysis

A microplate based protocol for measurement of endoglucanase activity has been reported to perform as well as the standard protocol [24]. Due to the elevated number of samples needed to be screened in characterization of segregating ears of maize, we decided to use this published protocol. Base line levels of endoglucanase activity measured by the CMC-microplate method in different genotypes of non-transgenic corn were established and

are presented in Fig. 4C. Due to availability of seed, B73 was selected as the genotype to use as a negative control. Since P198 and P199 lines have constitutive and endosperm-specific promoter, we also tested background endoglucanase activity levels in separated embryo and endosperm tissues of B73. It can be seen in Fig. 4D that endoglucanase activities are comparable in both tissues. Once the background levels of activity were established, we tested 10 events of transgenic line P199, analyzing up to 8 seeds per event. Seeds were imbibed overnight and hand dissected to remove embryo and pericarp. Isolated endosperms were used for TAEP extraction and CMC-microplate endoglucanase assay. B73 controls were processed in the same way. Figure 5A shows the results compiled for endoglucanase activity in P199. It can be seen that different seeds from different events have different levels of endoglucanase activity. It can also be seen that certain events, such as P199-35 has higher activity levels than the other events. In order to prove that high endoglucanase activity correlated with the presence of the transgene, we isolated DNA from corresponding embryos of the samples used in Fig. 5A, and performed PCR analysis using glucanase-specific gene primers. Figure 5B shows a representative result, using as example line P199-35; it shows that, in general, presence of the transgene correlates well with higher levels of endoglucanase activity measured by the CMC-microplate based method. Seeds lacking the transgene showed lower levels of endoglucanase activity, comparable to the B73 control. Up to this point this method seemed to be adequate and high-throughput, both desirable properties for an assay.

Degree of hydrolysis of CMC and gel permeation chromatography

To further characterize the nature of the endoglucanase activity present in transgenic line P199, positive extracts identified by the CMC-microplate based method were pooled and analyzed using traditional CMC hydrolysis and DNS reducing sugar determination. Figure 6A shows the hydrolysis profile of 1% CMC using commercial cellulase, extracts from P199 and B73. The transgenic maize TAEP (P199) had substantially higher enzyme activity in hydrolyzing the CMC than did wild type maize (B73), which indicated that bacterial glucanase was effectively expressed in the seeds of transgenic maize (P199). The Sepharose CL-2B chromatograms of the CMC and enzyme-hydrolyzed CMC (~10% hydrolysis) are

shown in Fig. 6B. At the 10% hydrolysis of CMC, the large molecules of CMC were hydrolyzed into small molecules with a narrow distribution both by cellulase and enzyme extracted from kernels of transgenic maize (P199). This result suggested that the enzyme extracted from kernels of transgenic maize (P199) had high endo enzyme activity, which was similar to cellulase, and higher compared to B73 maize.

Indicators of the non-adequacy of CMC-based assay for measuring recombinant endoglucanase activity in transgenic maize

Encouraged by the results obtained from CMC-hydrolysis assays we decided to establish the temperature effect on the recombinant glucanase. Protein extracts from lines P199 and B73 were incubated at different temperatures and assayed for endoglucanase activity using the CMC-microplate based method. Results presented in Fig. 6C show that there is no obvious temperature effect on endoglucanase activity of either P199 or B73 extracts. This result was unexpected, as endoglucanases, like most enzymes, have a response curve for temperature. The fact that the endoglucanase activity was still higher in P199 than in B73 led us to believe that there might have been initial differences in reducing sugars in both lines, independent of the presence or absence of CMC in the sample.

To confirm that this was the case, we decided to test several samples that had been screened by PCR (insert in Fig. 7A) for their endoglucanase activity after separating the protein from the extract by ammonium sulfate precipitation. We first carried out the assay as we had been performing it up until now, which resulted in levels of activity between 0.3 and 1.5 nmol of reducing sugars/ μ g TAEP/min. However, after protein precipitation, the activity levels were markedly diminished when incubated with CMC or when reducing sugars were measured without CMC. These observations confirmed that something was not right with the assay. Different seeds, transgenic or not, seemed to have initial levels of reducing sugars that were not predictable. To further confirm that the elevated levels of reducing sugars in TAEP extracts was not related to endoglucanase transgene, we tested several transgenic kernels expressing a variety of transgenes such as the B subunit of the heat labile enterotoxin (LT-B) of *Escherichia coli*, β -glucuronidase (GUS) or the green fluorescence protein (GFP). Figure 7B shows that non-glucanase lines such as GUS or GFP expressing transgenic lines

also show high levels of reducing sugars in the presence or absence of CMC. The non-discriminatory nature of this assay led us to conclude that it was not adequate for the characterization we were trying to achieve.

Endoglucanase activity based on methylumbelliferyl cellobioside hydrolysis

Due to the problems encountered with CMC-based assay, we decided to try another published procedure [11] for characterization of recombinant endoglucanase activity in transgenic plants. This method measures endoglucanase activity by release of a fluorescent molecule, 4-methylumbelliferone, upon hydrolysis of substrate 4-methylumbelliferyl cellobioside (MUC). Initial tests showed that background level using blanks and non-transgenic B73 were comparable to fluorescence levels for P198 and P199 reactions without incubation at the specified temperature for the reaction. After incubation at 50°C, the fluorescence detected in samples of transgenic lines P198 and P199 was significantly different from the blank and non-transgenic control. To further test if the enzymatic activity measured by this assay, we studied the response to different temperature treatments. Figure 8B shows that transgenic extracts of P199 showed maximum activity at 50°C, while B73 extracts showed lower activity and baseline across all temperatures. This result was encouraging, as it showed that the enzyme responsible for cleaving the exogenous substrate, 4-methylumbelliferyl cellobioside, was sensitive to temperature treatments. Figure 8C shows, however, that certain non-glucanase lines such as non-transgenic Hi II and transgenic GUS line P56 showed higher MUCase activity than P199-2 tested side by side.

MUCase hydrolysis is not a direct method of measurement of endoglucanase activity, as the natural substrate for endoglucanase is glucan, which is much larger than MUC. Endoglucanase activity estimated by this method is an indirect measure and is based on the fact that cellulose degradation occurs by concerted action of three enzymes: endo-1,4- β -glucanase, exo-cellobiohydrolase, and β -glucosidase [9]. The substrate used in this assay, MUC, presents much homology to the substrate for β -glucosidase. As these three enzymes act in synergy, it is conceivable that if endoglucanase activity is higher, there will potentially be more substrate available for the other two enzymes. This method has been used several times in the last five years to characterize endoglucanase activities of recombinant forms

produced in several plant species such as tobacco [10, 11], potato [12], Arabidopsis [13], rice [15] and maize [16, 17]. However, none of these studies reports recombinant endoglucanase production in seeds.

Concluding remarks and future work

Production of cellulolytic enzymes in the maize biomass has been considered as a significant way to reduce costs of bioethanol production [16, 17]. Published work on this approach has focused mainly on expressing the recombinant enzymes constitutively in the maize tissues, and assays for cellulolytic activity have been done using corn leaves or stover. In this work we have generated corn for expression of a bacterial endoglucanase under constitutive or seed-specific promoters. Even though molecular data (Southern blots and PCR) confirm the successful integration and inheritance of the transgene in maize plants, enzyme assays to characterize the recombinant protein have not been conclusive. Initial CMC zymograms and reducing sugar determination after CMC hydrolysis and Sepharose gel-chromatography suggest that the transgenic lines have enhanced endoglucanase activity. However, quantification by a microplate-based method based on the standard IUPAC assay for endoglucanase activity was unsuccessful in confirming our observations. We have approached several of the challenges presented by this method in order to avoid reporting misleading results. The CMC-based method for endoglucanase quantification seems to be non-specific and indicates that some transgenic lines, not related to an endoglucanase transgene, also have enhanced CMC-hydrolytic activity. Precipitation of protein from total aqueous extractable protein extracts might be a good option to consider if further utilization of this method is desired.

We also used published procedures using MUC as a substrate and fluorescence detection of cleaved 4-methyl-umbelliferone. This method has been used to characterize cellulolytic activity of recombinant endoglucanases. From our observations, this method might be a better option for optimization, even though its measurement of endoglucanase activity is indirect. High MUCase activity observed in non-glucanase transgenic lines (Fig. 8C) is not encouraging, but future work needs to include a larger number of transgenic endoglucanase

lines screened and compared against non-transgenic controls or non-glucanase transgenic lines.

Even though the major application of this work is related to the end-utilization of transgenic tissue for ethanol conversion, molecular and enzymatic characterization of transgenic lines is critical and required before taking any project further. Due to the great interest in heterologous production of endoglucanase enzymes we thought we could characterize the transgenic lines directly by measuring endoglucanase activity. Now that we have encountered many problems in doing so, our strategy for characterizing these lines will change. We plan to go back to basic DNA and RNA level characterization. Utilizing PCR at the DNA level will help us identify transgenic individuals (plantlets for P198 and seeds for P199) to work with. RNA level characterization will allow us to confirm that the transgene is being transcribed and that we can detect the mRNA in the tissue of interest, especially for transgenic line P198 in which the transgene should be constitutively expressed. With confirmed expression of the transgene we will then re-visit the characterization of the enzymatic activity. Our conclusion from CMC-based and MUC-based assays have led us to revisit our original approach of using a CMC-zymogram for detection of recombinant endoglucanase activity. We have already invested some work in establishing native gel electrophoresis protocols and transfer of proteins to a substrate containing gel for staining with congo red. Due to previous observations using a simplified dot-blot zymogram, TAEP extracts are suitable for this type of assay. However, some optimization might be needed for seed specific expressors.

The implications of publishing results from an assay that is not specific or robust can be very misleading and non-beneficial to the scientific community. Our results presented in this work help highlight the importance of validating results obtained from assays, even from previously published protocols. Our observations also raise important questions about the presence of reducing sugars in transgenic seeds, though we have not addressed the issue enough to make any conclusions about it. Our recommendation for recombinant endoglucanase characterization in plants includes further steps in protein purification, such as precipitation to separate the protein from extract containing endogenous reducing sugars, and verification of results with alternate methods of analysis.

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AA   H  T  N  D  T  M  T  V  T  S  A  K  D  L  V  A  K  M  T  N

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AA   D  K  Y  D  P  E  D  T  A  I  P  E  L  F  E  H  L  N  E  L

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Figure 1. Codon optimized sequence of EgI gene for expression in maize. The nucleotides highlighted in black have been changed in mEgI for maize codon preference.

		1		50
EGI	(1)	MNSKKIGAMIAAAVLSLIVMTPAATRKIVQRQ TRNSSTAVENSAADESET		
mEGI	(1)	-----	MRVLLVALALL	
		51		100
EGI	(51)	ENVVPSQ THTNDTMTVTSAKDLVAKMTNGWNLGNTMDATAQGLGSEVSWL		
mEGI	(19)	ALAASAT	SHTNDTMTVTSAKDLVAKMTNGWNLGNTMDATAQGLGSEVSWL	
		101		150
EGI	(101)	PLKVTTNKYMIDMLPEAGFNVLRI	PVSWGNIHIDDKYTSDPAWMDRVQEI	
mEGI	(62)	PLKVTTNKYMIDMLPEAGFNVLRI	PVSWGNIHIDDKYTSDPAWMDRVQEI	
		151		200
EGI	(151)	VNYGIDNGLYVILNTHHEEWYMPKPSEKDG	IEEIKAVWAQIADRFKGYD	
mEGI	(112)	VNYGIDNGLYVILNTHHEEWYMPKPSEKDG	IEEIKAVWAQIADRFKGYD	
		201		250
EGI	(201)	EHLIFEGLNEPRLRGEAEWTGTSEAREI	INEYEKAFVETVRASGGNNGD	
mEGI	(162)	EHLIFEGLNEPRLRGEAEWTGTSEAREI	INEYEKAFVETVRASGGNNGD	
		251		300
EGI	(251)	RCLMITGYAASSAYNNLSAIELPEDSDKLI	ISVHAYLPYSFALDTKGTDK	
mEGI	(212)	RCLMITGYAASSAYNNLSAIELPEDSDKLI	ISVHAYLPYSFALDTKGTDK	
		301		350
EGI	(301)	YDPEDTAIPELFEHLNELFISKGIPVIVGE	FGTMNKENTEDRVKCLELYL	
mEGI	(262)	YDPEDTAIPELFEHLNELFISKGIPVIVGE	FGTMNKENTEDRVKCLELYL	
		351		400
EGI	(351)	AAAAKYDIPCVWWDNYARIGNGENFGLMNRADLEWY	FPDLIETFKTYAEK	
mEGI	(312)	AAAAKYDIPCVWWDNYARIGNGENFGLMNRADLEWY	FPDLIETFKTYAEK	
		401		
EGI	(401)	DPASAE	----	
mEGI	(362)	DPASAE	KDEL	

Figure 2. Protein sequence of mEGI. Bacterial signal peptide of EGI was deleted (crossed-out text) and replaced by the signal peptide of the maize 27 kD γ -zein protein (normal text) in mEGI. Shadowed text shows amino acid sequence is conserved in both versions. Bold sequence KDEL in mEGI was added for retention in the endoplasmic reticulum.

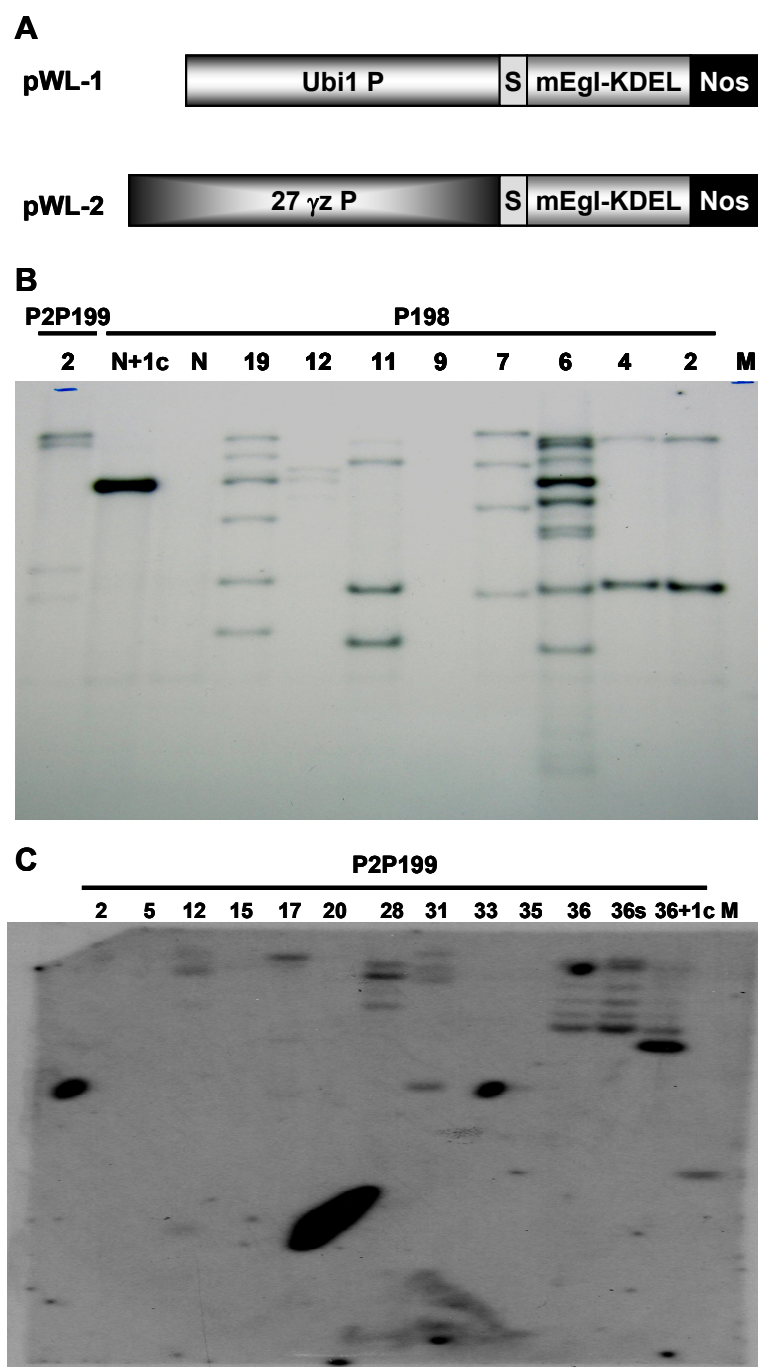


Figure 3. Schematic representation of constructs used for plant transformation (A) and Southern blots of transgenic lines P198 (B) and P199 (C).

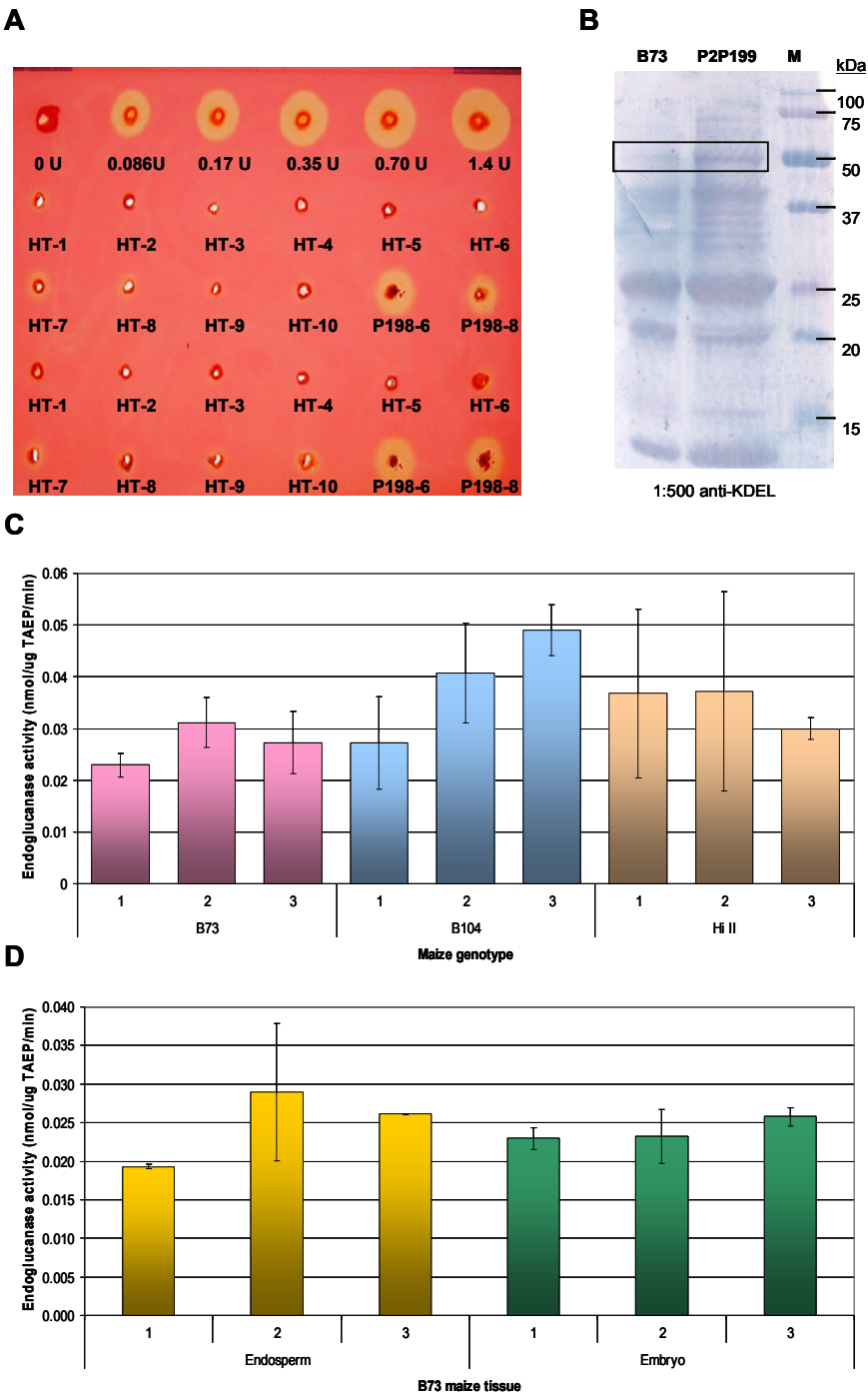


Figure 4. Zymogram showing carboxymethylcellulose (CMC) hydrolysis in P198 callus extracts (A), Western blotting using anti-KDEL antibody (B) and endoglucanase activities of wild type maize measured using CMC as a substrate and reducing sugar determination.

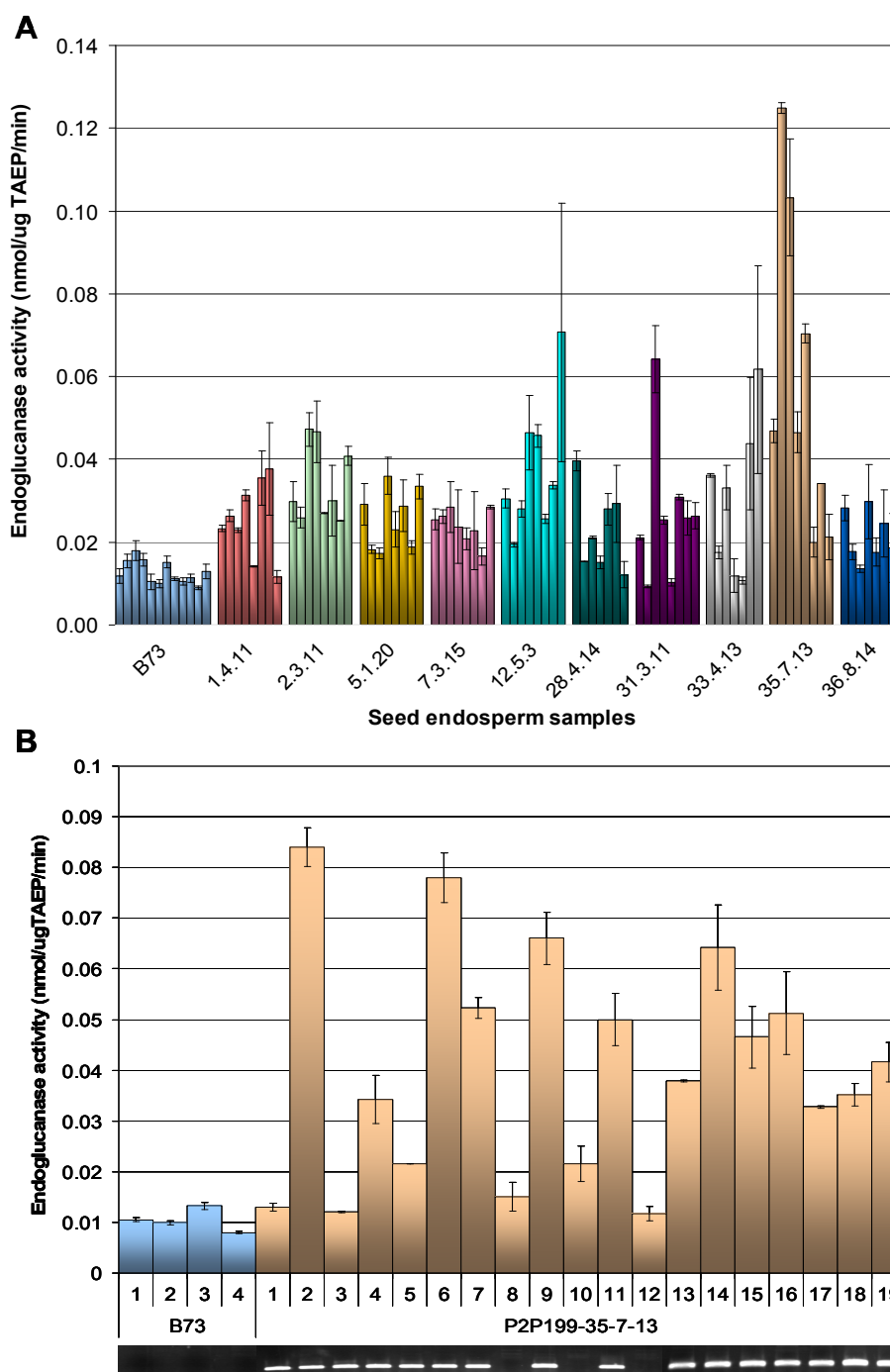


Figure 5. Endoglucanase activities of transgenic maize kernels of line P199 measured using CMC as a substrate and reducing sugar determination (A and B), and PCR product for individual transgenic kernels using mEgI-specific primers (B).

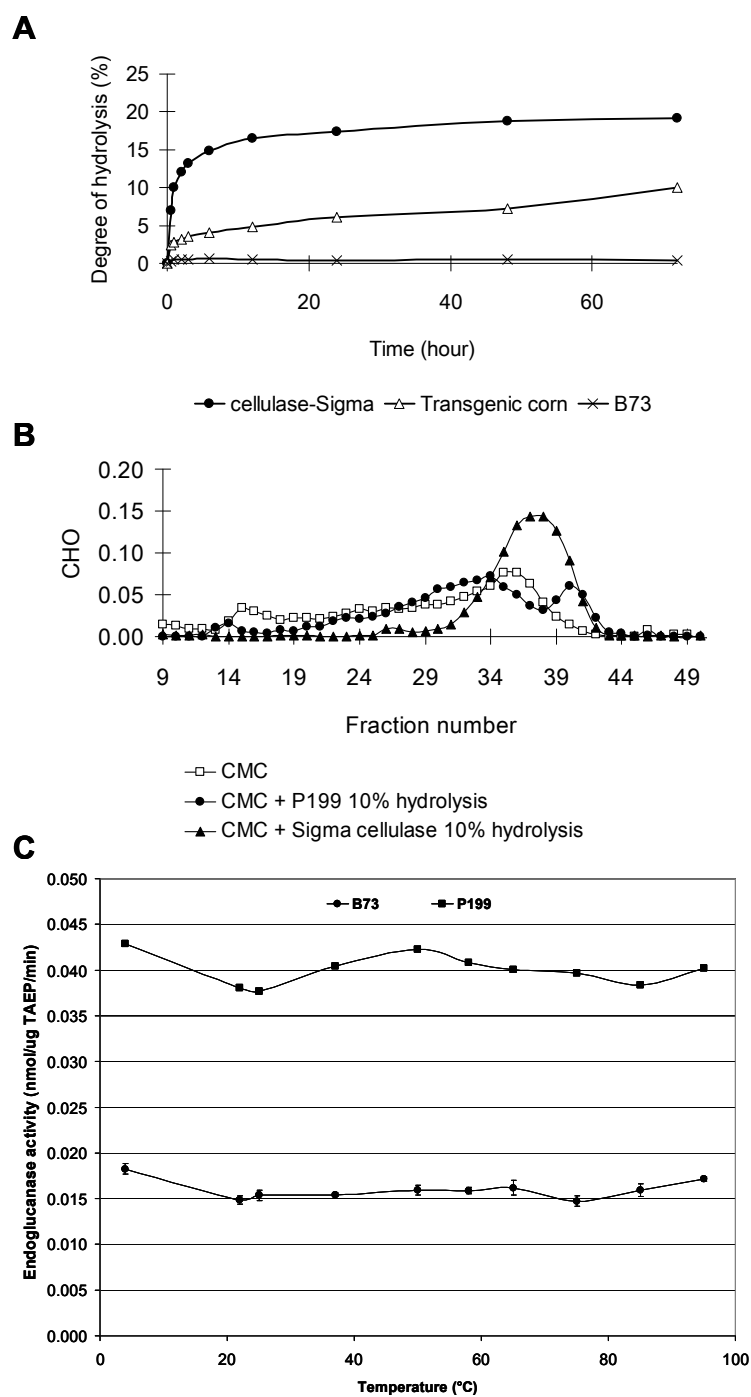


Figure 6. Carboxymethylcellulose hydrolysis (A), hydrolysis profile (B) and enzyme stability of protein extracts from line P199 (C).

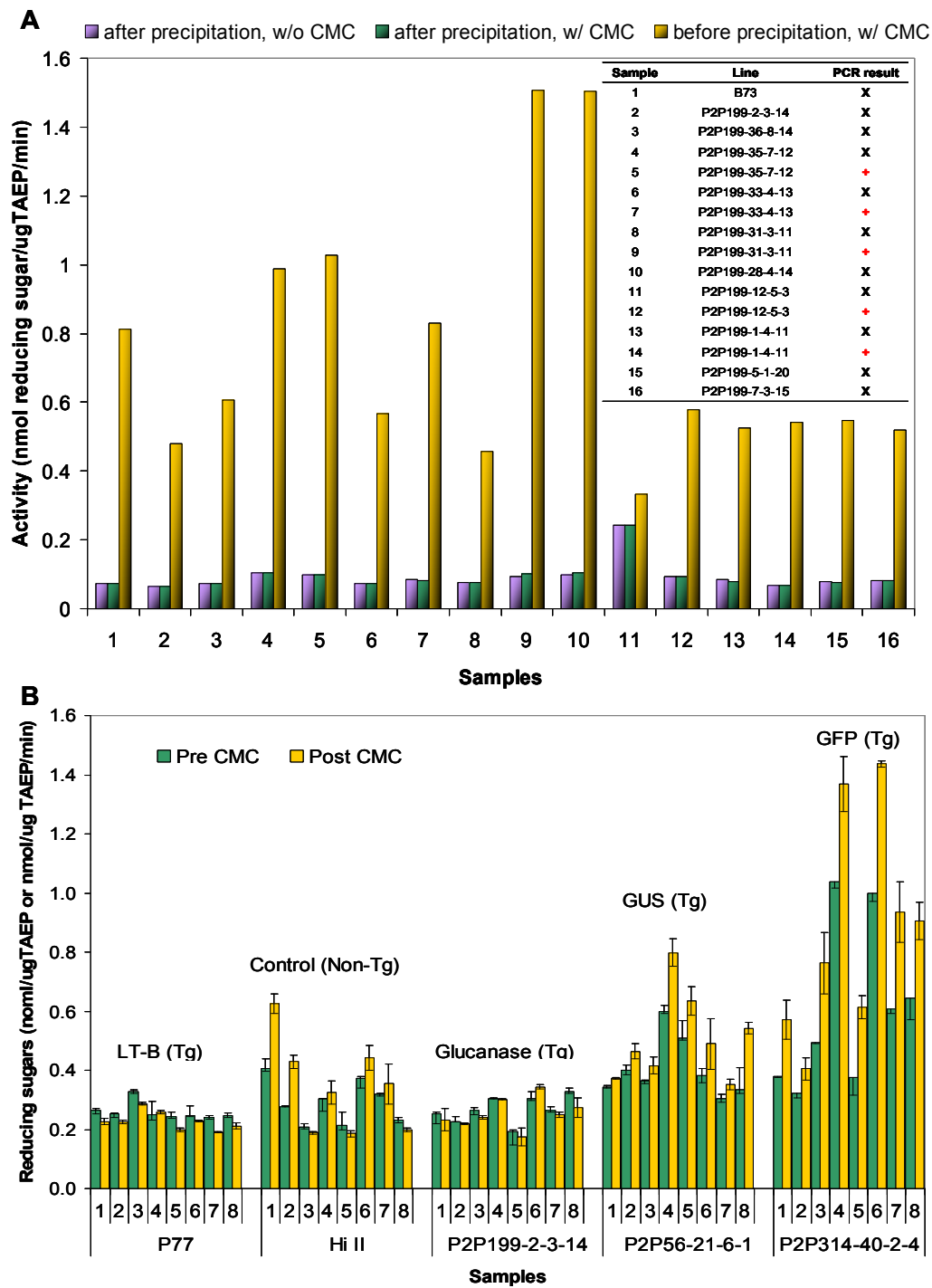


Figure 7. Endoglucanase activity measured after protein precipitation for PCR screened seeds of transgenic line P199 (A) and reducing sugar determination of non-glucanase maize (B).

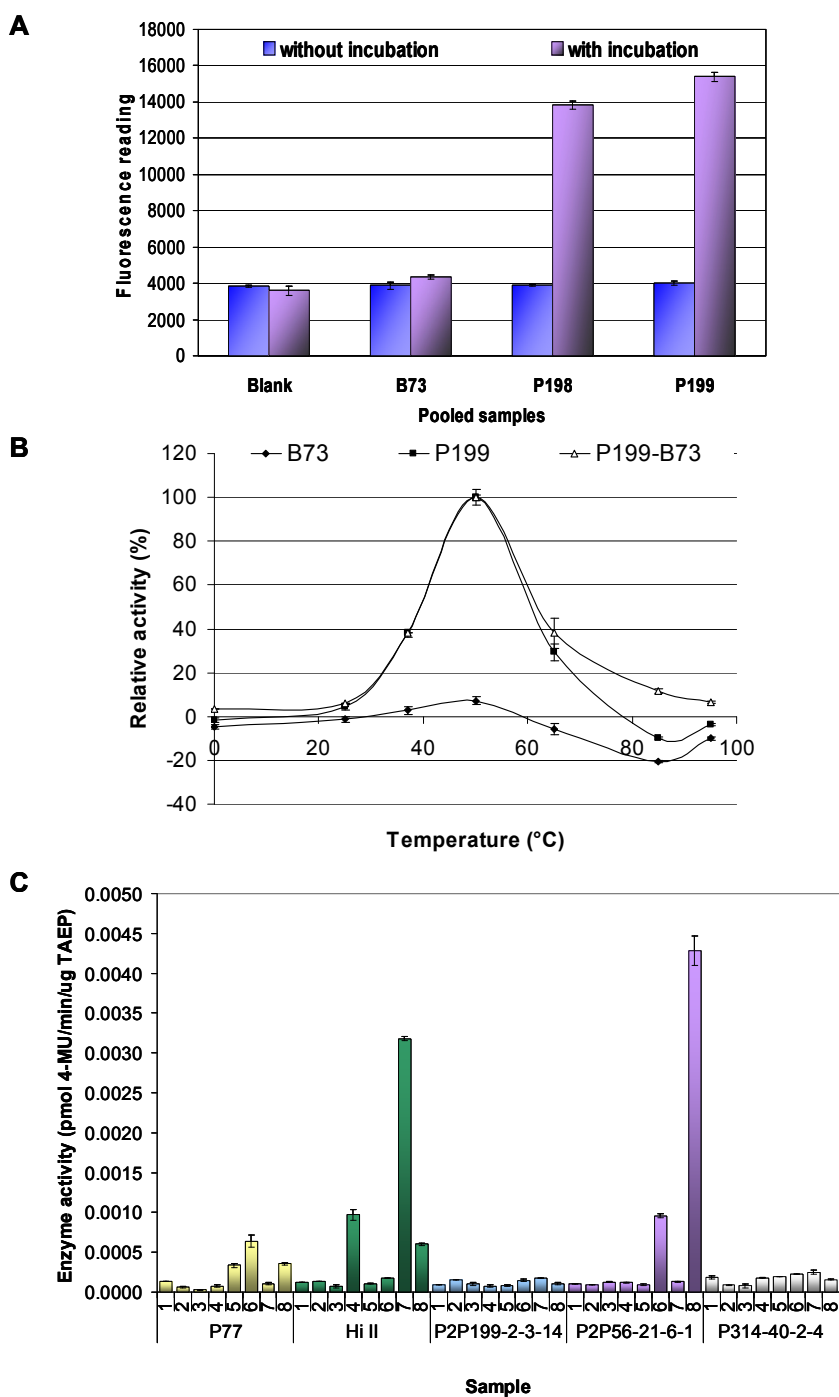


Figure 8. Endoglucanase activity measured using methylumbelliferyl-cellobioside (MUC) as a substrate (A), Thermal stability of protein extracts from transgenic line P199 (B) and MUC assay for of non-glucanase maize lines (B). P77, transgenic maize expressing the B subunit of the heat labile enterotoxin of *Escherichia coli*. Hi II, non-transgenic maize. P2P56, transgenic maize expressing β -glucuronidase. P314, transgenic maize expressing the green fluorescent protein.

CHAPTER 7: GENERAL CONCLUSIONS

This dissertation is a compilation of studies that focused on fundamental and practical considerations for use of maize as a platform for production of recombinant proteins. The work presented here involves several aspects of plant biotechnology including a review of tools available for plant genetic engineering, the utilization of protein fusions to study recombinant protein trafficking within plant cells, the bioprocessing of transgenic corn for downstream applications of recombinant proteins and byproducts, the utilization of maize endosperm tissue culture system for potential use as a tool in gene expression analysis, and the characterization of transgenic maize expressing industrial enzymes for biofuel production.

Genetically engineered crops have revolutionized agriculture and have made the transition from research settings to fields all over the world. Plant molecular biologists and genetic engineers use rapidly evolving technologies, molecular tools, genomic information and nanotechnology to engineer plants with more precision. In Chapter 2 we review the major plant transformation methods, discuss their strengths and limitations, and focus on a number of research areas that are likely to be used for designing and creating the new generation of transgenic crops in hopes of meeting the world demands for food, feed, fiber, and fuel.

The *Escherichia coli* heat labile enterotoxin B subunit (LT-B) has been used as a model antigen for the production of plant-derived, high-valued proteins. In Chapter 3 we studied the targeting properties of the bacterial LT-B protein and its native signal peptide (BSP) in maize and Arabidopsis. We used a series of translational fusions of LT-B with the green fluorescent protein (GFP) as a visual marker, with various combinations of signal peptides for transient and stable analysis in plant cells. We presented evidence that shows that LT-B::GFP fusion proteins assemble and fold properly retaining both antigenicity of LT-B and fluorescing properties of GFP when expressed in maize and Arabidopsis. The LT-B::GFP fusions and BSP-GFP were detected in both starch and fiber fractions of stably transformed maize seeds. We also show confocal microscopy evidence indicating that the proteins accumulate in secretory system of Arabidopsis and maize, in a signal peptide-dependent

fashion. Based on biochemical analysis of starch fractions and confocal microscopy, we conclude that LT-B fusion have a dual localization in maize kernels, which can be a species- or tissue-specific occurrence. Our results provide important insights for further understanding the heterologous protein trafficking mechanisms and developing effective strategies for the production of recombinant proteins in plants and future use of LT-B as a carrier molecule for other antigens.

Due to rising interest in using plants as biofactories for large-scale production of industrial enzymes, nutrition products and vaccines, in Chapter 4 we have studied the fractionation by wet milling of transgenic corn expressing the B subunit of the heat-labile enterotoxin of *Escherichia coli* (LT-B), a potent antigen and candidate for oral vaccine. Expression of LT-B in seed endosperm tissue by an endosperm-specific promoter has been shown to be effective in protecting mice against toxin challenge. We compared wet-milling of LT-B corn using two steeping treatments, traditional steeping (TS, 0.2% SO₂ + 0.5% lactic acid) and water steeping (WS, water only). LT-B distribution among the fractions was similar for both steep treatments, resulting in enrichment of functional LT-B in fine fiber, coarse fiber and pericarp fractions by concentration factors of 1.5 to 8 relative to the whole seed. Overall recovery of LT-B protein from WS treatment was > 1.5-fold greater than that from TS treatment, which indicates that TS has adverse effects on recovery of functional LT-B. From our analysis we conclude combined with endosperm-specific expression of LT-B, wet-milling enables enrichment of high-value proteins in low-value fractions, such as the fine fiber, and the utilization spent fractions for traditional uses such as biorefining starch to biofuels and industrial chemicals.

In Chapter 5 we presented the work done towards establishing and characterizing a tissue culture of maize endosperm cells derived from Hi II hybrid. Owing to the great importance of maize endosperm tissue to humans, research of maize has always included studies of maize seed development. However, the nature of endosperm tissue and lengthy growth season has prompted several groups to establish endosperm tissue cultures. Our work in Chapter 5 describes such a culture initiated from an elite genotype used routinely for maize transformation. Morphological analysis of the cells shows differentiation into aleurone-like and starchy endosperm-like cell types. Starchy endosperm cells of the

endosperm callus culture (ENC) have active starch biosynthetic enzymes and synthesize starch. ENC cells also transcribe and produce zeins. We present transient analysis of gene expression that reflects the dual nature of the cells of this culture, as they express embryo/aleurone promoter-driven, as well as zein-promoter driven constructs. We also show that the system described is useful in analysis of transgenic maize lines derived from Hi II transformation, which can be induced to form endosperm callus. The stability and continuous growth of this culture makes it a powerful tool for future applications in maize biotechnology.

Production of cellulolytic enzymes in maize biomass for ethanol production is an area of great interest nowadays. In Chapter 6 we present work done towards characterization of two lines (P198 and P199) of transgenic maize that express an endo-1,4- β -glucanase (mEgI) from *Ruminococcus albus* under constitutive or seed specific expression, respectively. We provide evidence for stable integration of the transgene using Southern blotting. We show that constitutively expressed mEGI enzyme was active in transgenic callus using a carboxymethylcellulose (CMC)-zymmogram assay. We also present the efforts towards implementing and using a microplate-based CMC assay for quantification of endoglucanase activity in transgenic maize seeds of line P199. Even though gel permeation analysis of CMC and enzyme-treated CMC show that the transgenic protein extracts can hydrolyze CMC to different degrees, temperature profile of the enzyme extracts and high levels of CMC-activity in non-glucanase maize lines suggested that the CMC method is not appropriate for characterization of recombinant glucanase activity in transgenic corn. We also present efforts towards using a fluorescent assay (MUCase) based on the hydrolysis of 4-methylumbelliferyl β -cellobioside and detection of fluorescent 4-methylumbelliferone to substitute the microplate-based CMC-assay. Further work is needed to establish the adequacy of MUCase assay for characterization of transgenic corn expressing recombinant endoglucanase, as preliminary analysis also show high levels of MUCase activity in non-glucanase corn. The potential applications of maize as a biofactory for cellulase enzyme production highlight the importance of relying on a robust method for characterization of our glucanase lines. Further work involves screening on the basis of gene integration and RNA transcription, while investigating novel assays for recombinant glucanase activity assays.

Since the intended end use of these lines is for bioethanol production, I suggest that the material be tested in pilot studies that directly measure the effect of the transgenic material in ethanol yield.

The work presented in this dissertation crosses several aspects of fundamental and applied science, fields that inevitably go hand in hand when using plants for human benefit. Looking across all topics discussed here, we can summarize our conclusions as follows. Combining traditional breeding and genetic engineering, we have more tools available to more precisely design commodity and specialty crops that will help meet the world's demand for food, feed, fiber and fuel production from plants. Production of recombinant proteins in plants continues to be of great interest in pharmaceutical, nutritional and industrial applications. The B subunit of the heat labile enterotoxin (LT-B) of *Escherichia coli*, a potent antigen and successful oral vaccine, can be expressed as a protein fusion with proteins as large as 27kD, a desired property for future use of LT-B as a carrier molecule. Our work identifies the role of the signal peptide of LT-B (BSP), a bacterial signal peptide, in subcellular targeting in plants. Since very little is known about how plants process bacterial proteins, this finding is significant in understanding that bacterial signals can function and be processed in plants, in a similar way as they are in bacteria, as BSP delivers proteins to the secretory pathway of plants. Transgenic maize expressing recombinant LT-B can be fractionated by wet-milling, resulting in enrichment of LT-B in the fine fiber fraction. Currently a low value fraction, LT-B enriched fine fiber can be used for direct oral delivery, processing or purification of LT-B, while spent fractions can be used for traditional uses such as oil extraction from germ or biorefinery of starch. Our study is the first to report effects of traditional steeping (with lactic acid) in recovery of LT-B by wet-milling. Based on our results, we recommend that water steeping be used instead, as it results in higher recovery of the recombinant protein. We have also described the establishment and characterization of a maize endosperm callus culture derived from Hi II maize genotype. We have shown that at this stage, the culture is not a useful tool for transient analysis of gene expression, but has other applications for studying endosperm transgene expression. The major utility of establishing an endosperm culture from Hi II, is having a culture derived from the genotype that is routinely used for transformation. Characterization of recombinant endoglucanase

activities in maize is not straightforward; published methods for endoglucanase assay CMC- and MUC- based did not give informative or consistent results. A robust method of analysis needs to be established for proper characterization of transgenic maize expressing recombinant endoglucanase for biofuel production. The combined results of our projects confirm the potential of using maize as a platform for production of recombinant proteins for nutritional, pharmaceutical or industrial applications.

APPENDIX 1: GENETIC ENGINEERING APPROACHES TO IMPROVE BIOETHANOL PRODUCTION FROM MAIZE

François Torney, Lorena Moeller, Andréa Scarpa and Kan Wang

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Abstract

Biofuels such as bioethanol are becoming a viable alternative to fossil fuels. Utilizing agricultural biomass for the production of biofuel has drawn much interest in many science and engineering disciplines. As one of the major crops, maize offers promise in this regard. Compared to other crops with biofuel potential, maize can provide both starch (seed) and cellulosic (stover) material for bioethanol production. However, the combination of food, feed and fuel in one crop, although appealing, raises concerns related to the land delineation and distribution of maize grown for energy versus food and feed. To avoid this dilemma, the conversion of maize biomass into bioethanol must be improved. Conventional breeding, molecular marker assisted breeding and genetic engineering have already had, and will continue to have, important roles in maize improvement. The rapidly expanding information from genomics and genetics combined with improved genetic engineering technologies offer a wide range of possibilities for enhanced bioethanol production from maize.

Introduction

The world energy demand is increasing steadily as the human population grows and economic development progresses. However, the current predominant energy source — the fossil fuel supply — is limited. This emphasizes the need to complement fossil-fuel-based energy sources with renewable energy sources, such as agricultural biomass [1]. Maize, currently one of two major biofuel crops in the United States, represents 31% of the world production of cereals and occupies a little over one fifth of the worldwide cereal-dedicated land [2]. In addition, maize is the second largest biotech crop grown world wide, after soybean, and a little over 10% of its cultivated surface is dedicated to biotech varieties [3].

To date, most maize genetic engineering has been performed using a few genotypes that are amenable to transformation and regeneration, but which do not always have the desired agronomic attributes [4] and [5] (see Figure 1). Improving our ability to introduce transgenes directly into inbred or elite genetic backgrounds is crucial for bioethanol production, because it reduces the time required for transgene introgression into elite maize lines. Other enabling technologies under development aim to improve the quality of transgene expression. These include tissue or developmental-stage-specific transgene expression, stringently regulated and induced gene expression [6], site-specific integration of the transgenes [7], expression of multiple transgenes, and gene stacking (i.e. adding transgenes sequentially in a genome) [8] and [9].

The net energetic benefit of using maize, mainly its starch component [1], for bioethanol production has been extensively reviewed [10] and [11] and is still debated among experts [11], [12] and [13]. Our focus will be on the various possibilities that genetic engineering can

offer to increase bioethanol production from maize (see Figure 2). This can be addressed from at least two angles: modifying biomass properties to reduce processing costs or increasing biomass yield and reducing agricultural inputs. We will review the latest studies on maize biology related to these aspects. Promising work in other species that could lead to improved bioethanol production in maize will also be discussed.

Genetic engineering to modify biomass properties

Two key parts of maize plants can be converted into bioethanol: the kernel, which is mainly made of starch, and the stover, which is predominantly made of lignin and cellulosic (cell wall) components. To convert them effectively into fermentable sugars for ethanol production, a range of approaches using genetic engineering have been explored. One strategy is to modify the characteristics and properties of starch or lignocellulose so that they can be converted more readily to the desired products. The other strategy is to introduce biomass conversion enzymes into plants so that they can aid the conversion process more effectively.

Starch composition

Today, ethanol from maize is produced almost exclusively from starch. The technologies and processes for deriving ethanol from maize kernel starch have been well-established since the 1980s [13].

Advances in understanding the starch biosynthetic pathway have been reviewed elsewhere [14] and provide new ways to redesign starch for specific purposes [15] and [16]. Starch is composed of two glucose polymers, amylose and amylopectin. In amylose, glucose units are linked in a linear fashion by α 1-4 linkages. Amylopectin, by contrast, is more branched and about 5% of its glucose units are linked by α 1-6 linkages. Normal maize starches contain about 20–30% of amylose and 70–80% amylopectin. The amylose/amylopectin ratio in starch affects its physical and physicochemical properties, such as gelatinization and recrystallization [17]. Alteration in starch structure can be achieved by modifying genes encoding the enzymes responsible for starch synthesis, many of which have more than one isoform [15] and [18]. Transgenic lines with modified expression of specific starch synthases, starch branching enzymes or starch debranching enzymes are being generated in attempts to produce starch granules with increased or decreased crystallinity, and thus altered susceptibility to enzymatic digestion (M James, personal communication). Another strategy is to reduce the energy requirements for the starch to ethanol conversion process. For example, gelatinization is the first step in bioethanol production from starch. It is conceivable that a modified starch with decreased gelatinization temperature might require less energy for the conversion process. Recent research showed that expression of a recombinant amylopullulanase in rice resulted in starch that when heated to 85°C was completely converted into soluble sugars [19].

Cell wall composition

Maize stover (leaves and stalks) constitutes a large part of agricultural biomass. Ethanol production from non-grain portions of plants is referred to as cellulosic or lignocellulosic

ethanol. Lignocellulose is composed of 30% hemicellulose, 44% cellulose and 26% lignin [20]. The structural crosslinking of these polymers presents a physical barrier to hydrolytic enzymes used in the ethanol conversion process, limiting its efficient usage for bioethanol production. Altering cell wall composition, mainly lignin, has long been contemplated as an option to enhance the efficiency of biomass conversion to ethanol [1].

Lignin is a vital component of the plant cell walls. It is responsible for the rigidity required for plant architecture, provides physical protection against pathogens and aids water transport in the xylem [21] and [22]. However, during the process of converting biomass into bioethanol, lignin limits the availability of polysaccharides to enzymes, therefore limiting the enzymatic degradability and digestibility of biomass. Maize brown midrib mutants (*bm*) with an altered lignin biosynthetic pathway have a naturally reduced lignin content and higher digestibility. Two transgenic approaches have successfully mimicked one of these mutant phenotypes (*bm3*) [23] and [24]. Piquemal *et al.* [24] used a maize caffeic acid *o*-methyltransferase (COMT) antisense gene construct and showed decreased COMT activity and lignin content in the transgenic maize. He *et al.* [23] obtained similar results using a sorghum *O*-methyl transferase antisense construct in maize, where transgenic plants showed increased digestibility. These studies show the feasibility of using plant transformation to modify the lignin biosynthetic pathway and to alter the lignin profile of maize.

As anticipated, altering plant lignin composition or content can lead to undesired agronomic consequences. Early studies showed that the *bm3* mutants were impaired in several agronomical traits; for example, grain and stover yields were reduced by 20% and 17%, respectively (reviewed in [25]). Additionally, *Arabidopsis* and alfalfa genetically engineered for an impaired lignin biosynthetic pathway showed dwarfism and/or flower color change [26] and [27]. Currently, more basic research is required to understand the lignin biosynthetic pathway and related areas. The future genetic engineering strategy should be a holistic approach to obtain maize with maximum digestibility in lignocellulose and minimum reduction in agronomic performance.

Biomass conversion enzymes

Although lignocellulosic feedstocks derived from corn stover could be used for conversion to bioethanol, two major limitations to the process are the costs of transport and processing of biomass. One solution is to produce microbial cellulase enzymes in the plant cells to facilitate the conversion of fermentable sugars *in planta* during the biomass to bioethanol conversion process [28]. Expression of the catalytic domain of the thermostable 1,4- β -endoglucanase (E1) of *Acidothermus cellulolyticus* in maize [29] proves the concept that maize can be used as a biofactory for cellulose-degrading enzymes. Even though expression of E1 has not achieved desirable levels, targeting the enzymes to specific subcellular compartments or tissues has shown to be effective in allowing the plants to accumulate higher levels of recombinant enzymes [30] and [31].

In addition to subcellular targeting of these enzymes, it is also important to express these cell wall degrading enzymes during appropriate developmental stages, rather than over the entire lifetime of the plants. Controlled expression would help to avoid undesired effects on agronomic performance such as lodging or susceptibility to diseases. A senescence-induced promoter might be used to drive cellulase expression in senescing maize. Ideally, the gene should be expressed at the end of the growing season or during post-harvest operations.

Other approaches include the use of plant endogenous genes to promote cell wall deconstruction; for example, expansins, a group of hydrogen bond-breaking proteins thought to loosen the cell wall during normal plant growth and development, might be such candidates [32].

Genetic engineering to improve biomass yield

Biomass yield is a complex trait. Although several biotech crop lines engineered for yield enhancement are currently being tested [33], the majority of genes involved in the trait remain elusive. Biomass yield increase and stabilization can be achieved through understanding and enhancing mechanisms such as stress tolerance [34], [35], [36] and [37] and carbohydrate metabolism [38].

Stress tolerance

Enhanced stress tolerance in plants has been achieved mainly through the manipulation of effector genes [39] (e.g. ion transporters, biosynthetic enzymes) and regulatory genes (e.g. transcription factors [40] or signal transduction components [41] and [42]) from maize itself, other plants or bacterial sources.

Transgenic maize expressing δ -endotoxins from *Bacillus thuringiensis* (Bt) is the classic example of genetic engineering for (biotic) stress resistance. This biotech maize is widely used in North America and constitutes 22 million hectares worldwide [3]. Among the strategies for next-generation insect-resistant crops are the expression of broad-spectrum insecticidal proteins from plants, from bacteria other than *B. thuringiensis* and novel proteins and peptide hormones from insects [43].

Although insect damage can account for as much as 10–20% of crop loss [42] environmental (abiotic) stress has been held responsible for 69% of crop loss [44]. Common denominators are found in response to several stresses, such as the accumulation of reactive oxygen species (ROS) with deleterious effects (e.g. DNA damage and/ or impairment of mitochondrial and chloroplast functions). Several excellent reviews addressing genetic engineering for abiotic stress tolerance have been recently published [^[34] and ^[35]] and here we will examine promising approaches centered on plant responses to oxidative stress.

Mitogen-activated protein kinases (MAPKs) are widely associated with the response to biotic and abiotic stress [45], and might be directly linked to the regulation of abscisic acid (ABA)-responsive antioxidant enzymes in maize [46]. Expression of a *Capsicum annuum* MAPK in rice and expression of upstream signaling components MAPK kinase kinases (MAPKKKs) from tobacco in *Arabidopsis* yielded increased tolerance to a range of biotic and abiotic stresses [47] and [48]. Our laboratory has demonstrated the benefits of this strategy in maize, where constitutive expression of *Nicotiana* protein kinase 1, a MAPKKK, enhanced freezing and drought tolerance in transgenic maize plants [41] and [42]. Other kinases as well as phosphatases also hold much potential in regulating signal transduction in response to stress [45].

De Block *et al.* [49] have successfully prevented the formation of ROS and consequently increased various stress tolerances in *Brassica napus* and *Arabidopsis*. Constitutive expression of the gene coding the antioxidant enzyme super oxide dismutase (SOD) in maize, led to increased tolerance to oxidative damage [39]. More recently, *Arabidopsis* plants with enhanced resistance to several abiotic stresses were obtained by overexpressing not a SOD

gene itself, but rather a microRNA involved in the fine regulation of two SOD genes, CSD1 and CSD2 [50].

Much of the study and engineering of plant stress resistance has been in model systems [34]. For instance, a particular class of transcription factors — the dehydration-responsive element-binding protein (DREB)/C-repeat-binding factor (CBF) — interact with the DRE/CRT *cis*-element of many stress-related genes and has been widely studied in *Arabidopsis* [35]. Constitutive overexpression of *OsDREB1A* and *OsDREB1B* in rice resulted in improved tolerance to drought, high-salt and cold stresses [51]. A recently cloned maize homologue, *ZmDREB1A*, enhanced cold tolerance when expressed in *Arabidopsis* [52]. Additionally, the overexpression of the *ZmCRT Binding Factor* increased cold tolerance in maize (reviewed in [40]). Results such as these indicate that many of the mechanisms used to enhance stress response pathways in model systems are applicable to maize and offer a key to reducing biomass and grain yield fluctuations, thereby ensuring steady production for biofuel.

Photosynthesis

As a C₄ plant, maize has a compartmentalized photosynthetic system that uses the phosphoenolpyruvate carboxylase (PEPC) as a primary carboxylase [53]. It has been reported that transgenic maize overexpressing PEPC has improved CO₂ fixation rate and compensation point, increased fresh and dry weight, enhanced leaf surface and stomatal density, as well as water stress resistance (reviewed in [54]). Additionally, recent work in transgenic tobacco showed that increased levels of fructose-1,6-bisphosphatase [55] and sedoheptulose-1,7-bisphosphatase [55] and [56], two Calvin cycle enzymes, significantly increased dry weight. Interestingly, expression of sedoheptulose-1,7-bisphosphatase also increased leaf area [56].

To adjust to the high planting density currently used in agriculture, modifying plant architecture becomes another way to improve photosynthesis [37]. It has been shown recently in rice that either reducing plant hormone brassinosteroid levels or the amount of the brassinosteroid receptors results in an erect leaf phenotype [57]. These erect leaf rice plants, obtained either through mutagenesis or genetic engineering, have enhanced biomass production and grain yield under conditions of high-dense planting with no extra fertilization. It is possible that the erect leaf plants are able to enhance photosynthesis by the leaves in the lower part of the plant owing to their altered architecture [58] or are able to reduce the ‘shade avoidance syndrome’ that is considered to cause stem elongation, early flowering and decreased grain yield in dense planting conditions [59] and [60].

Grain yield

In 2004, 11% of the maize grain produced in the United States was used to produce ethanol from starch. It is predicted that compared with the 12.87 billion liters of starch ethanol produced in 2004, in 2007 production will reach 20.44 billion liters [1] emphasizing the importance of starch production. As the ADP-glucose pyrophosphorylase (AGP) heterotetramer catalyzes the rate-limiting step in starch biosynthesis, it is usually referred to as a key enzyme in regulating sink strength in cereal seeds. Deregulation of AGP might lead to increases in plant sink strength and subsequent increases in seed and biomass yield [61], [62] and [63]. Smidansky *et al.* [61] transformed rice and wheat [63], using the maize

Shrunken2 gene Sh2r6hs coding for an AGP large subunit. Compared with control plants, both transgenic wheat and rice plants showed increased seed weight (increased by 38% and 23%, respectively) and increased biomass (increased by 31% and 22%, respectively). Recently a similar strategy in maize produced a 13% to 25% seed weight increase in AGP transgenic plants [64].

Conclusions

Genetic engineering technology presents undeniable potential for future agriculture and biofuel production, as described above. However, the acceptance of biotech-derived crops has met with skepticism and regulatory hurdles in many countries. One major public concern is the control of pollen dissemination for wind pollinated crops such as maize. Plastid genome transformation presents the advantage of limiting transmission of the transgene via pollen while preserving fertility of the plant and allowing higher transgene product production. Although transformation of plastid genomes has been achieved for a few plant species [65], it still remains to be demonstrated in maize. Male sterility offers an alternative approach to control transgene flow, an issue that will probably have a major impact on the development and routine use of biotech crops, in general, and of biofuel-destined crops in particular. Male sterility is a trait that is naturally present in certain lines but it can also be engineered. A recent demonstration of engineered male sterility used chloroplast transformation to produce completely male sterile tobacco plants [66].

It is now clear that multiple transgene strategies need to be developed to tackle complex traits, to engineer metabolic pathways and to combine the expression of different genes. Some studies have demonstrated the feasibility of such technologies [9] and [67], but more effort is needed to make them both applicable to bioethanol production and acceptable to the public. Indeed, the development of genetically engineered crops raises issues of legislation relating to how these technologies should be regulated and managed. Each country has its own legislation concerning plant biotechnology. Often the regulatory system lags behind the advancement of a technology. An integrated agri-biotechnology system for food, feed and fuel production is likely to be a challenge from the regulatory point of view, but will most certainly be the future for maize if it is to be bred for bioethanol production.

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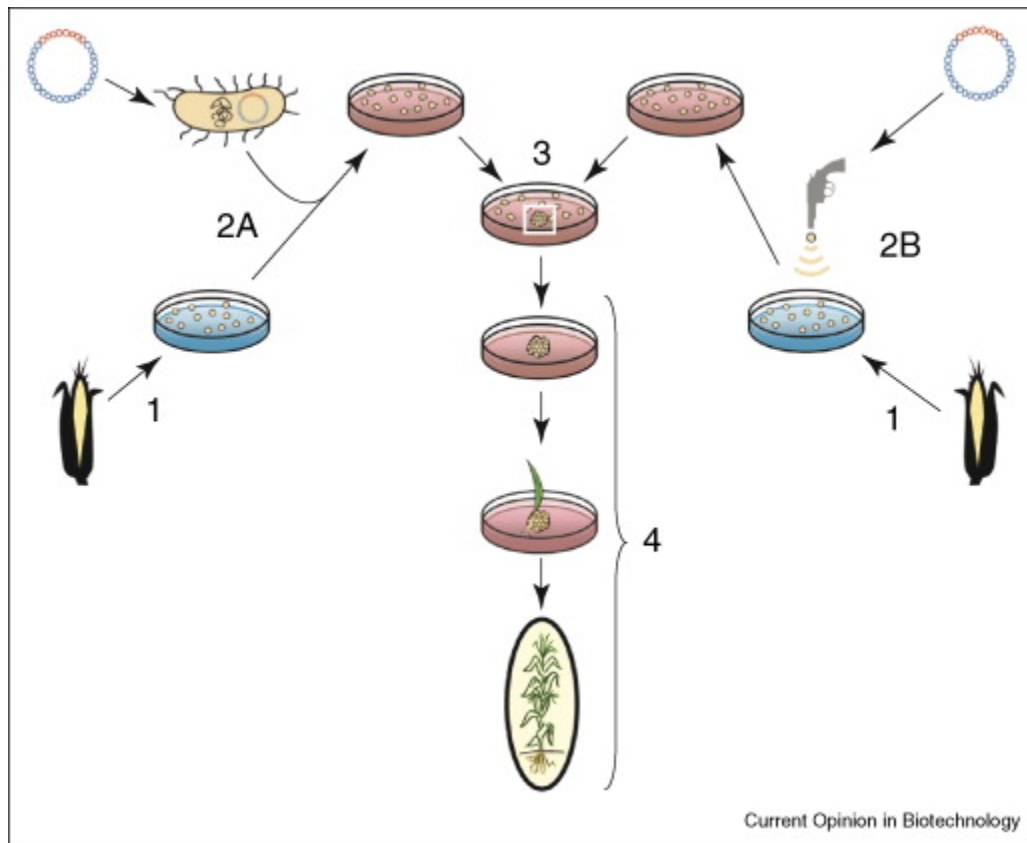


Figure 1. Two key approaches for the genetic transformation of maize. 1) Immature maize embryos are dissected from ears of corn harvested 11–14 days after pollination and placed on media containing nutrients and plant growth hormones (blue). The gene of interest can be introduced by one of two routes: 2A) the embryos are infected with an *Agrobacterium tumefaciens* strain that delivers the gene of interest and a selectable marker gene; or 2B) the embryos are bombarded with gold particles coated with one or more plasmids containing the gene of interest and a selectable marker gene. 3) Infected or bombarded embryos are placed on plant growth media supplemented with a selective agent (pink). Transformed cells expressing the selectable marker gene can proliferate and produce a callus mass (in square box). 4) The transgenic callus is cultured further and regenerated into mature transgenic maize plants that will subsequently be grown to maturity and analyzed.

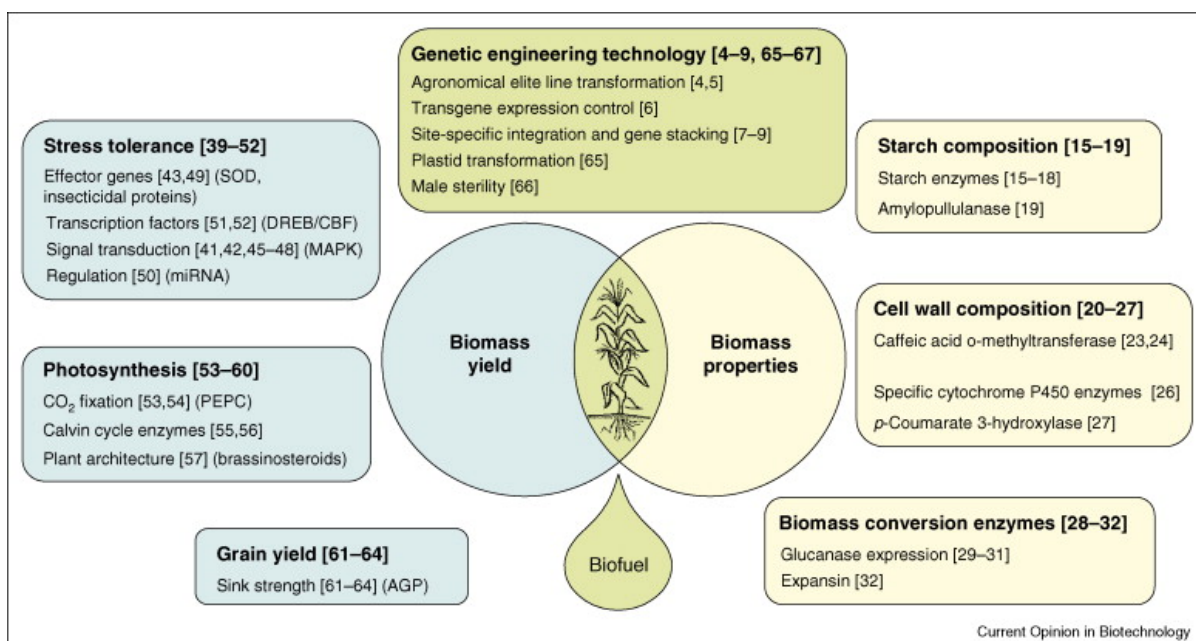


Figure 2. Possible approaches to enhance biofuel production from maize biomass. Two main routes for enhancing maize bioethanol production through genetic engineering are reviewed here: a quantitative and qualitative approach. The first aims to increase the biomass production per land area (i.e. the biomass yield and its stability). The second aims to alter biomass properties and composition to generate conversion process-friendly products for ethanol production.

APPENDIX 2: LOCALIZATION OF A BACTERIAL PROTEIN IN STARCH GRANULES OF TRANSGENIC MAIZE KERNELS

Rachel K. Chikwamba, M. Paul Scott, Lorena B. Mejía, Hugh S. Mason, and Kan Wang

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Abstract

The B subunit of *Escherichia coli* heat labile enterotoxin (LT-B) is a potent oral immunogen with potential for use as a vaccine, a carrier molecule to deliver antigens to gut-associated lymphoid tissues, and possibly an adjuvant to make coadministered vaccines more effective. LT-B produced in plants was shown to be functional and immunogenic in animals and humans. In this work, we show that maize-derived LT-B is strongly associated with starch granules in endosperm. Using immunogold labeling-electron microscopy, cell fractionation, and protein analysis techniques, we observed that LT-B protein could be detected both internally and externally in starch granules. This strong association confers an effective copurification of the antigen with the starch fraction of maize kernels, thermostability desirable in maize processing, and resistance to peptic degradation in simulated gastric fluid digests, an important attribute for an orally delivered antigen.

Introduction

The *Escherichia coli* heat labile enterotoxin B subunit (LT-B), a potent oral immunogen, has been used as a model antigen to demonstrate the feasibility of producing an effective oral vaccine in transgenic plants (1–3). Orally administered LT-B has been shown to elicit strong mucosal and serum antibody responses. LT-B could be used as an adjuvant, stimulating immune responses against coadministered antigens (4). In addition, LT-B's receptor-binding capacity in the gut makes it an ideal carrier molecule for the delivery of antigenic epitopes to the gut's mucosal system (5).

The likelihood of protein degradation in the stomach and uncertainty about the effectiveness of orally delivered antigens has raised concerns about the oral vaccination approach. The delivery of an oral vaccine in transgenic plant tissue could protect it from the low pH and digestive enzymes of the stomach as well as prolong the exposure of antigen to the gut's immune system. The subcellular location of any novel protein expressed in plants is also important for its accumulation, folding, and assembly, and, depending on its intended use, may have an effect on their functionality (6).

Proteins are commonly targeted to extracytosolic compartments in both eukaryotic and prokaryotic cells, which occurs by a variety of mechanisms. Translocation mechanisms usually involve the synthesis of a protein with a specific targeting signal and the recognition of this signal by the appropriate translocation machinery (7). Numerous bacterial and viral proteins have been produced in transgenic plants. Some proteins were transported into chloroplasts when they were fused to chloroplast transit peptide (8, 9). It is generally assumed that the subcellular destinations for those heterologous proteins produced in transgenic plants will be determined by the subcellular targeting information contained in the

protein, and an appropriate targeting signal is chosen based on the preferred destination for the protein. In this study, we determined the subcellular destination of the endosperm-expressed bacterial protein, LT-B, in transgenic maize kernels, when it was produced with either its native bacterial signal peptide or a plant signal peptide. In addition, we analyzed the practical significance of the observed LT-B distribution in maize endosperm.

Materials and Methods

DNA Constructs and Plant Transformation.

The LT-B gene cassettes were regulated by the maize endosperm-specific 27-kDa γ -zein promoter (10), tobacco etch virus translational enhancer leader sequence (11), and soybean vegetative storage protein terminator (12), as shown in Fig. 1. The synthetic LT-B gene (sLT-B) coding sequence was optimized for a compromise between maize and potato codon usage (1). Construct P77 contains the native LT-B bacterial signal peptide (1), and construct P81 contains the maize 27-kDa γ -zein signal peptide (10). Transformation of maize plants with constructs P77 and P81 (Fig. 1) was achieved by using the biolistic-gun method (13), and transgenic plants were grown to maturity in the greenhouse.

Fixation of Tissue for Immunolocalization.

Immature (23 d postpollination) and mature (dried) maize kernels were sectioned in fixative (0.1 M cacodylate buffer/0.5% glutaraldehyde/2% paraformaldehyde). Tissue blocks were incubated in fixative for 2 h at 4°C and rinsed three times in 0.1 M cacodylate buffer, 15 min each wash, on a rotating shaker. This step was followed by a succession of dehydrating treatments as follows: tissue blocks were rinsed with 50% ethanol for 15 min, followed by incubation with 70% ethanol for 2 h at room temperature. The 70% ethanol was removed and replaced with 95% ethanol for a 2-h incubation, followed by three 2-h incubations with 100% ethanol. Tissue blocks were then incubated in a gradually increasing concentration of white London Resin (LR white), starting with 1:3, 2:1, 3:1 (vol:vol) LR white to ethanol for 8–12 h each time, and finally with 100% LR white overnight. Incubation in pure LR white was repeated twice for 8–12 h each time, after which the tissue blocks were cast in gelatin capsules for 24–48 h at 60°C to polymerize. Ultrathin sections were cut and mounted on grids for scanning transmission electron microscope by the Bessey Microscopy Facility at Iowa State University.

Immunogold Labeling.

Kernel sections mounted on grids were incubated in TBS blocking buffer (0.05 M Tris, pH 8.3/0.85% NaCl, supplemented with 0.5% BSA/0.5% normal serum/3% dry nonfat milk) for 2 h at room temperature, followed by incubation of grids in primary antibodies, goat anti-LT-B polyclonal sera (Biogenesis, Bournewith, U.K.), rabbit anti-LT-B, rabbit anti-granule-bound starch synthase (GBSS) (S. Wessler, University of Georgia, Athens, GA), or rabbit anti- α -zein polyclonal sera at 37°C for 2 h. All primary antibodies were diluted 1:50 (vol:vol) in blocking buffer, with the exception of the rabbit antizein, which was diluted 1:100 (vol:vol), also in blocking buffer. Negative control grids were incubated in fresh blocking buffer. Grids were washed three times, 10 min each wash, in TBS buffer supplemented with 0.5% normal serum and 0.5% BSA, and incubated at room temperature in donkey anti-goat

polyclonal serum or goat anti-rabbit polyclonal serum conjugated to 12 nm of gold, diluted 1:20 and 1:50 (vol:vol), respectively, in TBS buffer supplemented with 0.5% normal donkey (for goat anti-LT-B grids), or normal goat serum (for rabbit anti-GBSS, LT-B, or zein grids), and 0.5% BSA/0.1% fish gelatin. Finally, the grids were washed three times, 10 min each wash, in distilled water and allowed to air dry before viewing under the scanning transmission electron microscope.

Starch Sample Preparation.

Starch was purified by using the procedure of White *et al.* (14), with the following modifications. Mature dry kernels were soaked in glass vials containing 2–4 ml of 0.45% $\text{Na}_2\text{S}_2\text{O}_5$ for 3 d. The pericarp and embryo were removed by using a razor blade. Endosperms were placed into a 50-ml conical tube with 10 ml of distilled water and homogenized with a rotor stator homogenizer. Homogenate was vacuum filtered through a 30- μM nylon filter (Spectrum Laboratories, Houston, no. 146506) cut to size in a side-arm flask, and the filtrate collected in a 50-ml conical tube placed under the filter assembly. Filtered homogenates were allowed to settle for a minimum of 1.5 h at 4°C. Water was removed from the sample by aspiration and the remaining starch water slurry transferred to a 2-ml tube. The slurry was centrifuged at low speed for 5 min. The starch was again washed three times with 1.5 ml of water, and one of three reagents, 75% ethanol, 95% ethanol, or 75% ethanol plus 3% β -mercaptoethanol. Samples were dried in a centrifugal evaporator (Speed-Vac, Savant). To determine the amount of LT-B in each kernel fraction, mature P77 kernels from an individual ear were used. LT-B-expressing and -non-expressing segregants were distinguished and separated by endosperm drilling and subsequent G_{M1} ELISA, as described (3). Endosperm from both groups were split in three parts, one portion for starch extraction for determination of starch-associated LT-B, the other two for LT-B, and total protein determination in the whole endosperm fraction.

Protein Extraction and Measurements.

Total proteins from kernel materials were extracted by using the following buffer: 25 mM sodium phosphate (pH 6.6)/100 mM NaCl/0.1% Triton X-100 (vol:vol)/1 mM EDTA/10 $\mu\text{g}/\text{ml}$ of leupeptin (wt:vol)/0.25 mM serine protease inhibitor Perfabloc SC (Fluka), at room temperature. Aqueous extractable protein was determined in all samples (drilled kernels, crushed endosperms, and starch) by using the Bradford assay (15). Total nitrogen determination and protein content conversion were performed by using a LECO CHN-2000 (LECO, St. Joseph, MI), according to AOAC Official Method 990.03.

Removal of Exogenous Proteins.

To remove exogenous proteins from purified starch or ground maize kernel, samples were incubated with 5 mM CaCl_2 solution containing 100 $\mu\text{g}/\text{ml}$ thermolysin (Sigma) at 64°C for ≈ 16 h (16). The reactions were terminated by the addition of EDTA to 20 mM. Samples were subsequently washed five times in distilled water to remove thermolysin and were then boiled in SDS sample buffer (17) (1 ml per 50 mg of starch) for 7 min at 95°C for Western analysis (3).

Determination of Thermal Stability.

To determine the thermal stability of maize-expressed LT-B, 50 mg of ground meal from P77 transgenic maize meal was wetted with 250 μ l of sterile distilled water, and an identical amount of nontransgenic maize meal was wetted in sterile water spiked with 1 μ g/ml LT-B. The wet meals were incubated at room temperature (25°C), and 37, 55, 65, or 85°C for 2 or 4 h. At the end of the treatment, 250 μ l of 2 \times protein extraction buffer was added to all samples, for a final concentration of 10 μ l of extraction buffer for every milligram of ground maize meal. All samples were shaken for 1 h at room temperature, and functional LT-B was quantified by G_{M1} ELISA, as described. Resistance to thermal degradation was determined by comparing the functional LT-B remaining in the treated samples to the functional LT-B in the samples not subjected to any heat treatment.

Simulated Gastric Fluid (SGF) Digests.

The SGF contained 3.2 mg/ml porcine pepsin (Sigma) in 34 mM NaCl, 0.7% HCl, as described (18). Twenty-five milligrams of transgenic maize meal containing 0.05 μ g of functional LT-B was incubated in 250 μ l of SGF. For controls, 5 μ g of recombinant LT-B (J. Clements, Tulane University, New Orleans) was added to 25 mg of nontransgenic maize meal and also incubated in 250 μ l of SGF. Incubations were done at 37°C for 30 s; 1, 5, 15, 30 min; or 2 h. The reactions were terminated by the addition of 50 μ l of 1.5 M Tris·HCl (pH 8.8), 200 μ l of 2 \times SDS sample buffer containing β -mercaptoethanol, and boiled at 95°C for 7 min. Fifty microliters of each treated sample was separated on an SDS/18% PAGE, transferred to a 0.45- μ m nitrocellulose membrane, and analyzed by Western analysis (3).

Results

An sLT-B was fused to DNA sequences encoding either the native LT-B signal peptide (Construct P77) or maize 27 kDa γ -zein signal peptide (construct P81), as described in Fig. 1. Twenty independent transformation events from each construct were evaluated for the expression of the LT-B gene in seed by using a ganglioside-dependent enzyme-linked immunosorbent assay that measures functional LT-B (3). The LT-B proteins produced by maize plants carrying either construct (P77 and P81) were structurally and functionally identical to the bacterium-derived LT-B protein. Oral immunization with maize-synthesized LT-B induced a strong immune response in BALB/c mice, protecting them from challenge with the *E. coli* LT and its homologue cholera toxin (3).

Immunogold labeling was used to determine the subcellular localization of LT-B within transgenic maize kernels. Ultrathin sections of 23-day-old immature kernels from P77 and nontransgenic control plants were processed as described in **Materials and Methods** and viewed by transmission electron microscopy. As shown in Fig. 2B, LT-B was detected exclusively in starch granules of immature kernels of P77 transgenic lines. No specific signal could be detected near or within cell walls, intercellular spaces, the endoplasmic reticulum (ER), Golgi apparatus, or secretory vesicles. No gold particles could be detected in the mature and immature kernels of nontransgenic maize and nontransgenic segregants (data not shown). For a positive control, we used the maize GBSS, an enzyme involved in starch biosynthesis and naturally found inside starch granules (19). Fig. 2C shows an identical localization pattern for the GBSS and LT-B, confirming the starch localization of LT-B in

transgenic maize lines. To rule out the possibility of artifactual gold accumulation in the starch as a result of the labeling process, we used the same procedure to determine the localization of the maize α -zein protein as another control. The zein proteins are the major seed storage protein in maize, comprising 50–60% of endosperm protein (10), and are localized outside of the amyloplast, the subcellular compartment in which starch granules are formed. As shown in Fig. 2D, α -zein protein was localized exclusively in the cytoplasm of the cell, and no gold particles could be detected in the starch granules, indicating that gold localization in starch was not a result of the labeling process in our hands. The α -zein was expected to localize in intact protein bodies, and the cytoplasmic localization observed here shows disruption of the protein bodies during maturation and/or processing.

To investigate whether the starch localization of LT-B protein requires its native signal peptide, immunolocalization of LT-B was carried out in LT-B-expressing P81 transgenic maize kernels, in which the bacterial signal peptide was replaced with the signal peptide from maize γ -zein protein (Fig. 1). As observed in P77 kernels, starch granule localization of LT-B was also detected in P81 kernels (data not shown). This result indicated that the native signal peptide of LT-B was not necessary for the starch localization of the LT-B protein in transgenic maize seed.

To determine whether the LT-B proteins were localized at the surface or inside of the starch granules, starch samples from mature transgenic seeds were collected and treated to remove surface proteins, and granule-associated proteins were analyzed on immunoblots. The approach was based on the fact that polypeptides within the starch granules are not susceptible to hydrolysis on treatment of intact granules with exogenous proteases (16). Starch samples were isolated from individual mature kernels of the transgenic maize line carrying construct P77, using three different levels of preparation stringency (75% ethanol, 95% ethanol, or 75% ethanol plus 3% β -mercaptoethanol) to remove external proteins. The starch samples were then treated with thermolysin (EC 3.4.24.27) for 16 h to remove remaining external proteins not embedded in the starch granules. The samples were then boiled to disrupt the starch granules, releasing embedded proteins. Duplicate SDS/PAGE gels containing equal amounts of protein were analyzed by Western blotting by using antibodies against LT-B, GBSS, or α -zein proteins. Zeins serve as convenient markers for extragranular protein contamination, whereas GBSS is an internal control for starch-bound proteins. As shown in Fig. 3, both LT-B and zein proteins could be detected in starch samples treated with 75 or 95% ethanol (lanes 2 and 3 of Fig. 3 A and B). However, only LT-B protein could be detected in starch samples subjected to the most stringent treatment with 75% ethanol/3% β -mercaptoethanol (lane 1, Fig. 3A). Neither protein was detected in the nontransgenic starch samples treated the same way (lane 4 in Fig. 3 A and B). Western analysis also shows the presence of the 60-kDa GBSS used as the positive control for starch-encapsulated proteins in all boiled and protease-treated samples, as expected (Fig. 3C, lanes 1–4). These results support the immunogold localization observation that LT-B is present within the starch granules of maize kernels.

Although LT-B protein was consistently detected in starch granules by two different sets of antibodies (goat anti-LT-B and rabbit anti-LT-B followed by the relevant secondary antibody gold conjugates), Western blots and G_{M1} -dependent ELISA analysis of ground kernels (3) and intact starch granules indicated that the LT-B protein is also present in the soluble fraction of the endosperm. These observations suggested that LT-B has similar

properties to some starch biosynthetic enzymes, such as starch synthase I, which are present in both soluble and granule-bound forms in amyloplast of maize endosperm (19). We conducted further analyses of the distribution of LT-B between the starch fraction and the soluble fraction of the endosperm.

Table 1 shows the partitioning of total protein and LT-B between starch and the soluble fraction. Total protein determined by the combustion analysis method was 8.784% of ground endosperm material, in the range of average protein content (8.7%) in endosperm of a normal dent maize variety (20). Total aqueous extractable protein (TAEP) determined by Bradford assay was 0.23% of the ground endosperm. The functional pentameric form of LT-B determined by the G_{M1} -dependent ELISA is 0.0002% of the ground endosperm. The total protein determined in the starch fraction was 2.028% of the dry starch weight, \approx 4-fold reduction from the 8.784% observed in the ground endosperm. It is also noted that the ratio of total protein vs. TAEP in starch (2.028 vs. 0.027) is 75-fold, whereas this ratio in endosperm (8.784 vs. 0.234) is 37-fold. However, in the starch fraction, despite the significantly smaller amount of TAEP, LT-B constitutes 0.00013% of the dry weight, more than half the amount observed in the whole endosperm fraction (0.0002%). These data are a quantitative indication that more than half of the LT-B in the endosperm is starch associated.

Fig. 4 illustrates that LT-B is detectable in the soluble fraction of endosperm cells prepared without disrupting starch granule structure, and that it is also externally associated with the purified starch sample. Fig. 4A shows that LT-B constitutes a much larger proportion of total protein in starch ($>0.006\%$) than in endosperm ($>0.002\%$). A similar observation was made on TAEP. Fig. 4B shows that LT-B constitutes $>0.45\%$ of TAEP in starch, whereas in endosperm, LT-B is $<0.1\%$ of TAEP. These observations are indicative of a strong association between LT-B and starch in endosperm tissue.

To determine the practical importance of the intricate association of LT-B with the maize tissue matrix, we examined its thermostability and resistance to proteolytic degradation by the biologically relevant gastric enzymes and small intestinal enzymes. It has been demonstrated that the B subunit dissociates between 66 and 78°C (5). Gelatinization of starch is an essential procedure in maize processing for humans and occurs above 65°C, depending on the length of exposure. We incubated wet transgenic maize meal and starch at 37, 55, 65, or 85°C for 2 or 4 h. Soluble bacterium-derived LT-B was added to an equal amount of nontransgenic maize meal or starch and subjected to the same treatment. Percent retention of functional LT-B in treated samples was established from the ratio of G_{M1} -captured LT-B in treated to untreated samples (room temperature, 25°C). Fig. 5 shows the results after incubation of samples for 4 h; the data for 2-h incubation were similar but are not presented. Fig. 5A shows that functional LT-B increases with increase in incubation temperature up to 55°C. This upward trend could possibly be a result of increased release of LT-B from the corn matrix. These data are consistent with previous observations that incubation of transgenic maize meal at elevated temperature enhanced LT-B extraction (R.K.C., unpublished work). Functional LT-B declines at 65°C; LT-B in ground meal declines slightly to 97.2%, whereas the soluble LT-B declines more markedly to 82.1% (Fig. 5A). At 85°C, 5.49% functional LT-B is retained in the transgenic meal, but no functional LT-B can be detected in the soluble LT-B samples. Similar trends in functional LT-B retention were observed in starch (Fig. 5B). Transgenic starch appears to have a similar protective effect on LT-B to the transgenic maize meal. This effect is markedly pronounced when the starch

samples were incubated at gelatinization temperatures (65 or 85°C) for both 2-(data not shown) and 4-h treatments, with a much higher protective effect apparent after incubation for the 4-h period (Fig. 5B). In starch samples, however, no functional LT-B is detected after incubation at 85°C for 4 h. It is noted that at temperatures above 65°C, the purified starch gelatinizes more readily than the whole meal. The retention of functional LT-B in its functional form is therefore achieved in both transgenic starch and transgenic whole meal but is stronger in the latter.

Fig. 6 shows the results of Western blot analysis indicating the presence of monomeric LT-B in samples after SGF treatment. Twenty-five milligrams of transgenic maize material contained 0.05 µg of functional pentameric LT-B determined by G_{M1}-dependent ELISA (Table 1, Fig. 6 B1 and B2). The control was 25 mg of nontransgenic maize material, to which 5 µg of bacterial LT-B was added (Fig. 6 A1 and A2). Soluble LT-B added to nontransgenic maize meal was completely digested by pepsin within 5 min of SGF treatment (Fig. 6, lanes 2, 3, 4, and 16), whereas LT-B present in transgenic maize meal resisted SGF digestion up to 15 min (Fig. 6, lanes 6–9 and 11). It is important to note that we were not able to determine the exact amount of monomeric LT-B in transgenic maize material and bacterial LT-B control. However, it was reported that pentameric form of LT-B constituted ≈40% of total LT-B protein (2). Thus, we deduced there were ≈2 µg of pentameric LT-B in the control samples, 40× more LT-B compared with transgenic material.

The result of trypsin and chymotrypsin digests also showed that LT-B in general was resistant to degradation by small intestinal enzymes, but maize-associated LT-B was more resistant to these enzymes (data not shown). These data, together with our immunological results obtained from animal studies comparing maize-derived LT-B with bacterial LT-B (3), strongly suggest that the maize starch-associated LT-B has significant resistance to gastric enzymatic digestions and led to increased antibodies responses in mice (3).

Discussion

We observed in this work the localization of a bacterial protein in the starch granules of transgenic maize seed. These observations are contrary to our expectation, in which LT-B protein in transgenic maize kernels would be strictly ER targeted and ultimately secreted to the cell wall and extracellular spaces (21). This result led to further analyses of LT-B partitioning in maize endosperm. Data from these analyses strongly suggested that LT-B protein was detected not only internally but also externally of starch granules in maize endosperm. Interestingly, the immunogold localization did not reveal LT-B localized outside starch granules. This discrepancy between the immunogold-labeling observation and the soluble extractable protein ELISA as well as Western analysis data could be explained by the possibility that antibody-reactive epitopes of LT-B in the soluble kernel fraction were sensitive to the processing procedures and/or reagents, whereas the granule-bound forms were protected from fixative and polymerization procedures of tissue preparation. Another possibility is that the LT-B protein level in the soluble fraction of the endosperm was beneath the sensitivity level of the immunogold detection technique.

Our immunolocalization observations suggest that a novel amyloplast-targeting mechanism may be present in plant cells. In enterotoxigenic forms of *E. coli*, LT-B is targeted to the periplasmic space (22), whereas in yeast, LT-B was shown to be ER targeted, assembled into pentamers, and retained within the endomembrane system (23). In plants, the

default pathway for proteins transported through the ER is secretion, although a protein's ultimate subcellular localization also depends on factors other than just the presence of a signal sequence, including topological information on the protein itself (24).

Although the import of nucleus-encoded proteins into chloroplasts has been studied extensively (25), the mechanism of import of nucleus-encoded proteins into amyloplasts has not been established (16). Chloroplast-targeted proteins are synthesized with transit peptides usually removed upon import into the chloroplast. Targeting to amyloplasts is thought to occur by a similar mechanism, because most nucleus-encoded starch biosynthetic enzymes have sequences reminiscent of chloroplast transit peptides (26).

The mechanism by which LT-B is sorted to starch granules is not clear. Judging by the migration of LT-B in SDS/PAGE, we believe maize-derived mature LT-B protein produced with either the bacterial signal peptide or the plant signal peptide was processed appropriately in plant cells (3) (data not shown). Because the maize-derived LT-B with either signal peptide was detected in starch granules, it seems likely that the starch targeting information must be contained on the mature LT-B protein itself. Current models of plastid targeting involve translation in the cytoplasm and subsequent import into the plastid. If LT-B is transported to starch granules via the ER, a mechanism for returning LT-B to the cytoplasm or a novel vesicle-mediated sorting pathway would be required. Transport of LT-B from the ER to the cytoplasm could involve retrotranslocation, whereby proteins are exported from the lumen or membrane of the ER into the cytosol (27). Retrotranslocation has been reported for the A subunits of the cholera and *E. coli* toxins (27–29). Whether the B subunits undergo a similar process is not determined.

Recently, the twin arginine translocase (tat) has been described, and this pathway commonly operates in eukaryotic thylakoid membranes and the prokaryotic plasma membranes (30). The novelty of this pathway is its unique ability to transport folded proteins through tightly sealed membranes without the hydrolysis of nucleotide triphosphate(s) at any stage in the translocation process. Proteins transported via this pathway have an essential twin-arginine motif in their signal peptides. The LT-B protein with or without the signal peptide motif does not have this feature. However, not all of the components of this pathway have been identified. The tat pathway has been shown to translocate multimeric proteins across membranes. It is possible that LT-B, a pentameric protein, is transported into the amyloplast by using a similar mechanism.

Few transgenic proteins have been analyzed in great detail for their subcellular localization in plants. Düring *et al.* (31) targeted immunoglobulin chains to intercellular spaces in transgenic tobacco by using the barley aleurone α -amylase signal peptide. This group observed localization of the assembled monoclonal antibodies in the ER and, unexpectedly, in the chloroplasts. Because no particular features of the immunoglobulins were expected to target the chloroplasts, they could not explain this observation. Similarly, our group observed LT-B localization in starch granules. It is possible that this association is facilitated by the affinity of LT-B to the galactose portion of the galactolipids on plastid membranes, because LT-B has a demonstrated affinity for galactose (5). The apparent starch localization of LT-B could well be random or explained by any of the pathways we describe. However, such unexpected observations hint at pathways and biochemical processes yet to be unraveled. Further investigation of the starch-targeting property of the LT-B protein by

using site-directed mutagenesis and reporter genes will contribute to the elucidation of mechanisms of protein translocation to the plastid.

There are several apparent practical advantages of expressing LT-B in maize endosperm. First, our data (Table 1) indicate that in this transgenic line, 1.3 and 2 g of LT-B can be obtained from 1 kg of starch and endosperm, respectively. Because most aqueous soluble proteins are lost in the starch purification process, the large amount of soluble LT-B copurified with starch fraction suggested a tight association of LT-B with starch. The copurification of LT-B with starch can reduce the presence of other undesirable endosperm proteins in the final product. Second, the internal and external localization of LT-B protein with maize starch granules has important implications on the effectiveness of a maize-based oral vaccine. Our work demonstrates that expression of LT-B in transgenic maize endosperm tissue conferred heat resistance at temperature and exposure periods where the starch is partially or completely gelatinized, and gelatinization of starch is part of the processing treatments to make maize meal more palatable for humans, such as in the manufacture of breakfast cereals and other snacks. In this work, the purified starch gelatinizes more readily than the whole meal, hence the increased reduction in functional LT-B retention in transgenic kernel-derived starch compared with the ground transgenic meal. Although it is not clear whether the starch-internalized LT-B is in multimeric (functional) or monomeric form, we showed earlier that mice orally immunized with this LT-B-expressing transgenic maize meal developed a higher serum and mucosal antibody response than mice immunized with nontransgenic maize meal mixed with an equivalent amount of bacterial LT-B (3). One of the major hurdles to oral immunization is the fact that antigens have to survive the severely low pH and degradative action of the gastric environment (32). We demonstrated here that maize-derived LT-B is more resistant to peptic cleavage in SGF digestion analysis. Vaccines encapsulated in or strongly bound to starch granules could be protected from the harsh environment of the stomach and possibly released slowly from the starch, resulting in prolonged exposure of the antigen to the gut-associated mucosal system. In addition, encapsulation of novel proteins in starch by using a domain in the LT-B protein as a targeting signal could greatly facilitate the protein extraction and purification process in maize.

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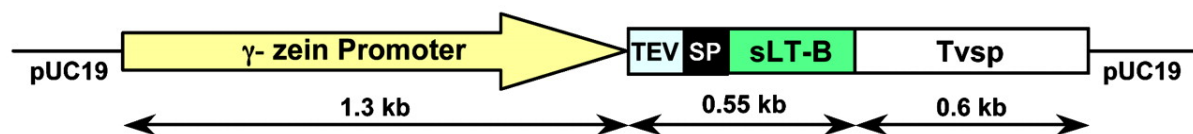


Figure 1. Schematic diagram of constructs P77 and P81 used for maize transformation to generate LT-B-expressing transgenic plants. Both constructs are in a pUC19 vector and contain the 27-kDa maize γ -zein promoter, tobacco etch virus (TEV) translational enhancer leader sequence, sLT-B, and soybean vegetative storage protein terminator (Tvsp). SP, signal peptide. P77, sLT-B fused to the bacterial signal peptide from LT-B; P81, sLT-B fused to signal peptide from the maize 27 kDa γ -zein protein.

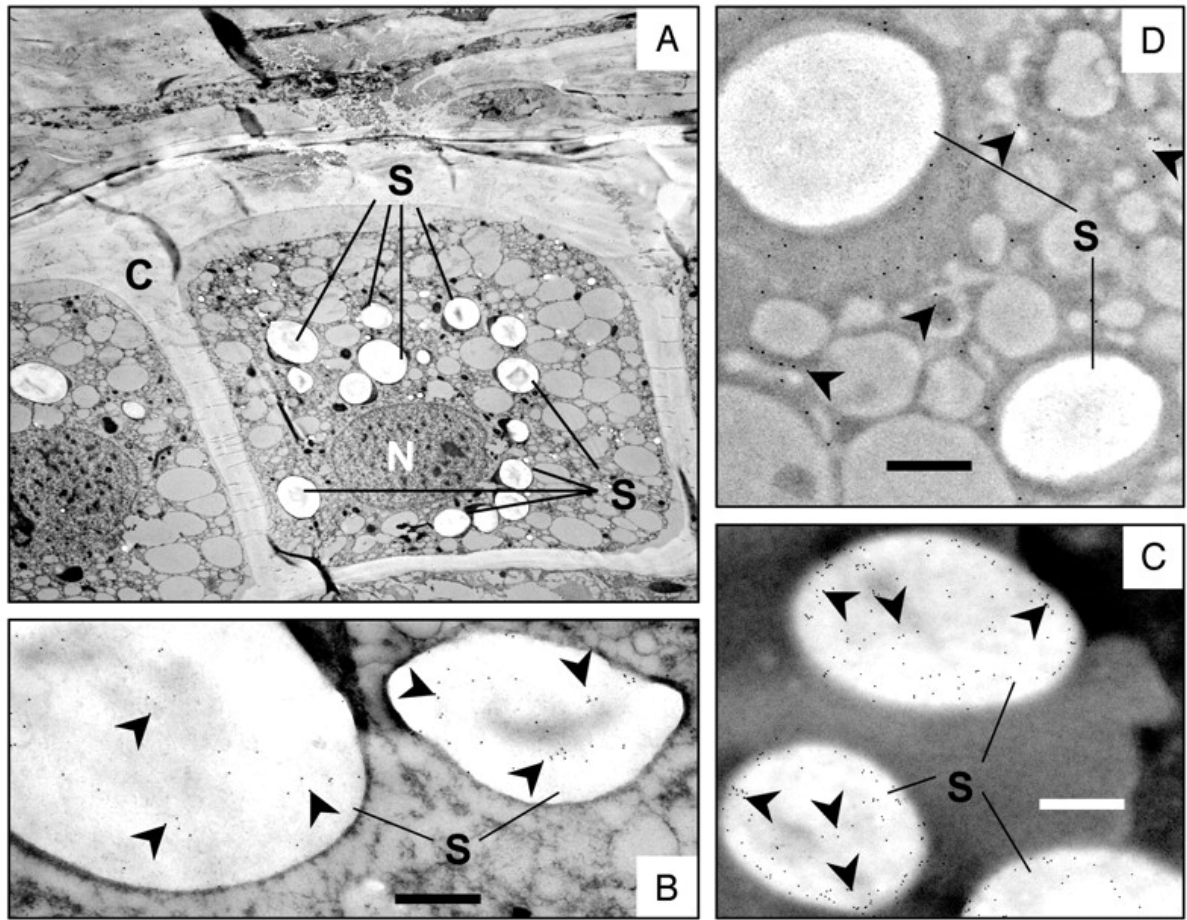


Figure 2. Immunolocalization of LT-B in immature maize kernels. (A) Section of maize kernel showing a cell beneath the aleurone layer. C, cell wall; N, nucleus; S, starch granules. (B) P77 transgenic kernels showing LT-B localization in starch granules. (C) GBSS localization in starch granules. (D) α -Zein protein localization in cytoplasm. Arrowheads indicate gold particles. (Bars = 500 nm at $\times 18,000$.)

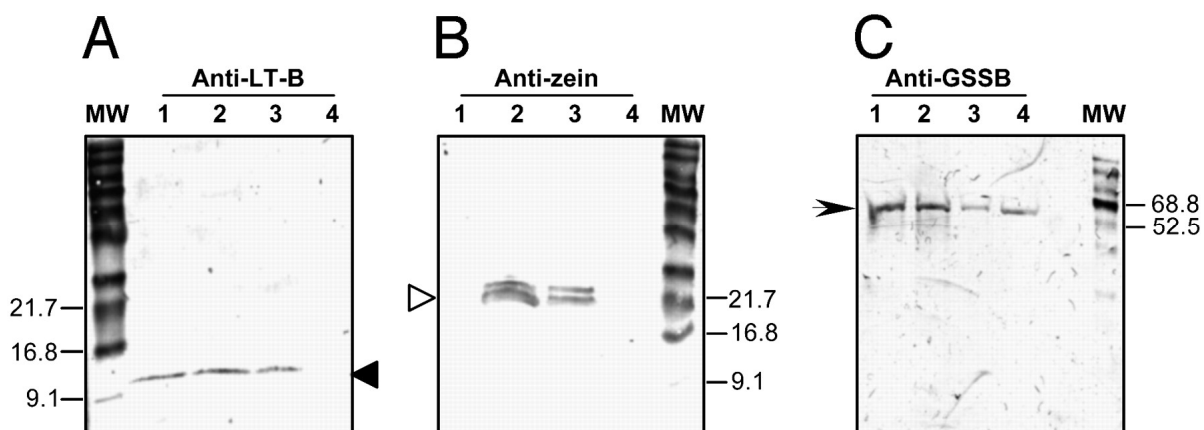


Figure 3. Western blot analyses of total proteins from starch samples after thermolysin treatment. The samples were separated on a SDS/12% PAGE, transferred to a 0.45- μ m nitrocellulose membrane, and probed with goat anti-LT-B antibodies (*A*), rabbit anti-zein antibodies (*B*), or rabbit anti-GBSS (*C*), followed by rabbit anti-goat or goat anti-rabbit alkaline phosphatase conjugate, respectively. P77 starch samples washed with 75% ethanol plus 3% β -mercaptoethanol (lane 1), 75% ethanol (lane 2), or 95% ethanol (lane 3), and B73 nontransgenic starch samples washed with 75% ethanol plus 3% β -mercaptoethanol (lane 4), respectively. Solid arrows in *A*, monomeric form of LT-B (11.6 kDa); open arrow in *B*, zein proteins (19 and 22 kD); arrowhead in *C*, GBSS (60 kD).

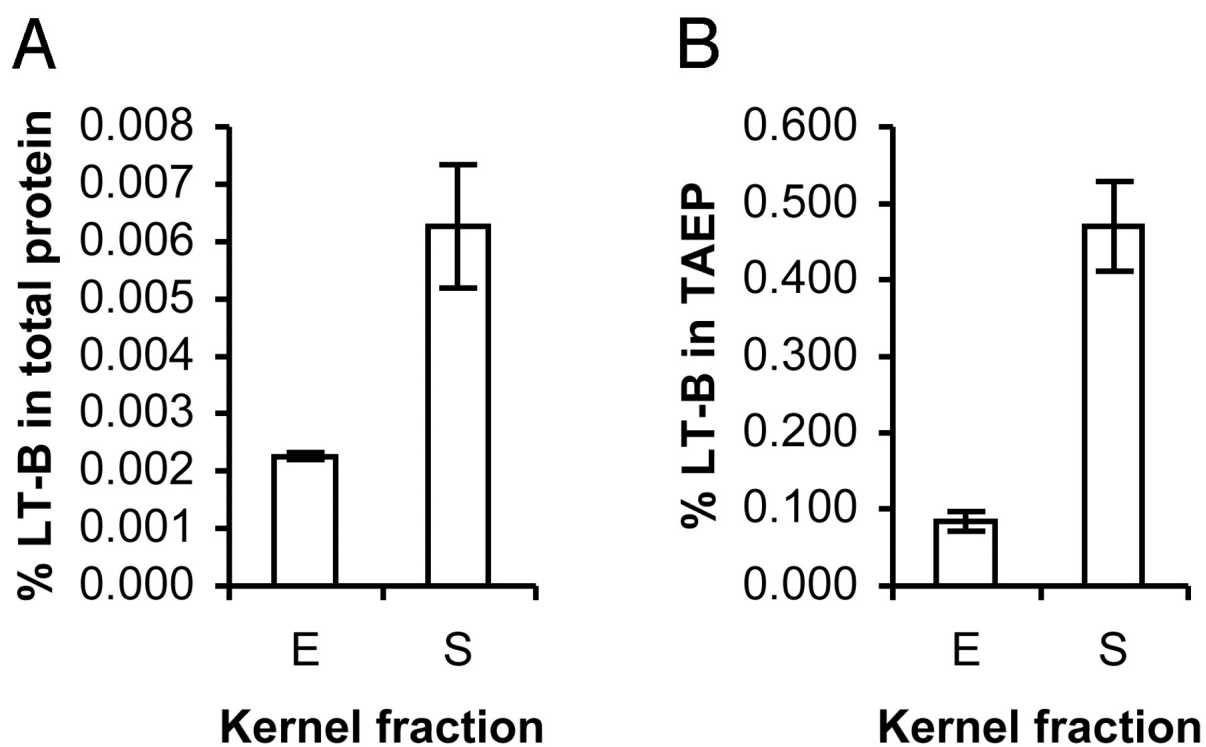


Figure 4. Percentage of LT-B in total protein (*A*) and TAEP (*B*) of maize kernel fractions. E, endosperm fraction; S, starch fraction.

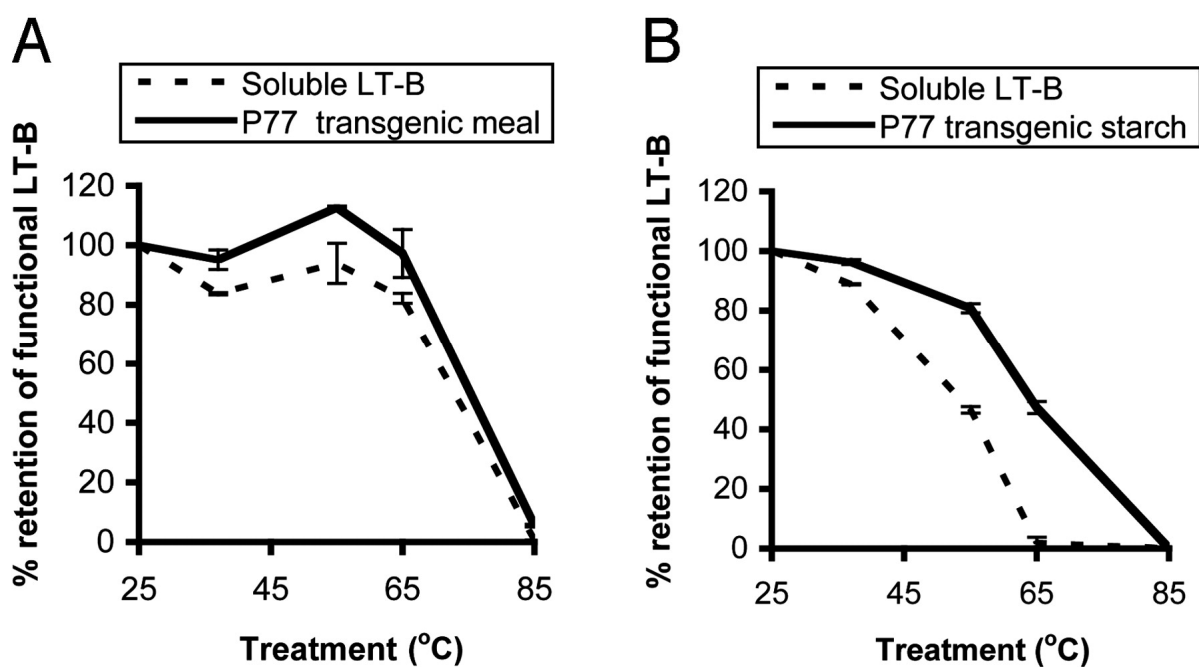


Figure 5. Retention of functional (G_{MI} -binding) LT-B in TAEP after 4 h of incubation at 37, 55, 65, and 85°C. (A) Ground maize meal. (B) Purified maize starch. Solid lines, transgenic maize line P77; dotted lines, nontransgenic maize control spiked with 1 µg/ml of bacterium-derived soluble LT-B.

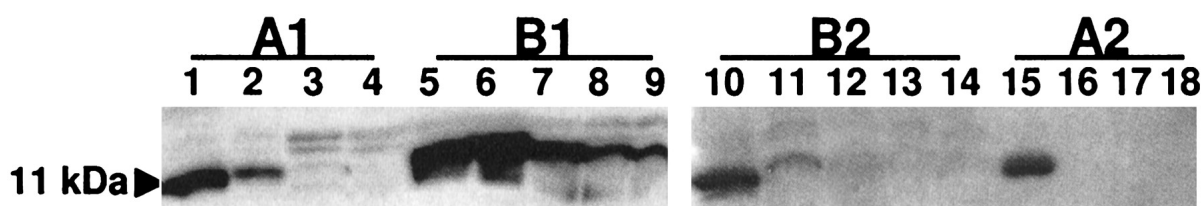


Figure 6. Western blot analysis of LT-B after gastric fluid digestion simulation. (*A1* and *A2*) Ground maize kernels mixed with bacterial LT-B. (*B1* and *B2*) Ground transgenic maize kernels. The samples were treated with pepsin at various length of period and separated on an SDS/18% PAGE and transferred to a 0.45- μ m nitrocellulose membrane and probed with rabbit anti-LT-B antibodies. Lanes 1, 5, 10, and 15, 0-min digestion (no digestion control); lanes 2 and 6, 30-s digestion; lanes 3 and 7, 1-min digestion; lanes 4 and 8, 5-min digestion; lanes 9, 11, and 16, 15-min digestion; lanes 12 and 17, 30-min digestion; lanes 13 and 18, 60-min digestion; lane 14, 120-min digestion.

Table 1. Percentage of total protein and LT-B in transgenic maize kernel fractions

Fraction	Total protein, %*	Total TAEP, %†	Total functional LT-B, %‡
Endosperm	8.784 ± 0.206	0.234 ± 0.031	0.00020 ± 0.00000
Starch	2.028 ± 0.053	0.027 ± 0.002	0.00013 ± 0.00002

Measured as milligram per 100 mg of ground material

* Total protein calculated from total N content, determined by the Dumas-modified combustion method by using a LECO CHN-2000 (AOAC Official Method 990.03)

† TAEP, determined by Bradford assay (15)

‡ Total LT-B from GM1-dependent ELISA (3)